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# The AMERICAN JOURNAL of MEDICAL TECHNOLOGY

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## HEMATOLOGIC OBSERVATIONS IN A FAMILY SHOWING COMBINATIONS OF SICKLE CELL, TYPE C, THALASSEMIA AND NORMAL HEMOGLOBINS\*

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In 1949 Pauling and his associates<sup>(15)</sup> introduced the concept that sickle cell anemia was a genetically controlled state in which the rate of hemoglobin synthesis was altered. Since that time, many investigators have contributed to the present knowledge of hemoglobin synthesis, and other types have been discovered.

To date there are ten known hemoglobin groups with new types being described every few months. In 1953 a committee was formed to adopt a uniform system of nomenclature, and it was decided that letters of the alphabet should be used with A to stand for normal adult hemoglobin, S for sickle cell hemoglobin, F for fetal hemoglobin, and all others that followed to be listed alphabetically beginning with the letter C.<sup>(21)</sup> The letter B was bypassed in order to avoid confusion because early workers had designated this letter to stand for sickle cell hemoglobin.

Approximately 99% of the hemoglobin found in the normal adult is termed Hgb A, the remaining fraction being Hgb F. Hgb F appears in the first red cells during fetal life and is gradually replaced by the adult pigment; nonetheless, 55% to 85% remains at the time of birth and traces may be detected as late as the third or fourth year.<sup>(4)</sup> Increased amounts of Hgb F may reappear in adults in certain pathological states when the body is in need of hemoglobin. Ordinarily, it is believed that its synthesis is inhibited by Hgb A. Detection of Hgb F in the laboratory is made easy by its unique resistance to denaturation by alkaline solutions<sup>(18)</sup> and by its electrophoretic mobility.

\* Scientific Products Foundation—Hematology, 1st Award, 1956. Read before First North American Conference of Medical Laboratory Technologists, Quebec, Canada, June, 1956.

Some investigators feel that Hgb S is found only when negroid ancestry is evident or cannot be ruled out. Its abnormal electrophoretic behavior was first noted by Pauling<sup>(14, 15)</sup> who also described the method by which it is believed that cells containing Hgb S are able to form the sickle shapes in the reduced form and the normal biconcave discs when oxygenated. In the reduced state, Hgb S is fifty times less soluble in a strong phosphate buffer than reduced Hgb A.<sup>(6)</sup>

A third abnormal pigment, Hgb C, was first discovered by Itano,<sup>(8)</sup> a coworker of Pauling. Its slow electrophoretic mobility toward the anode indicates that it is a more positively charged ion than either Hgbs A, F or S. When reduced, it is more soluble in phosphate buffer than reduced Hgb A or Hgb S.

Hgb D is very rare and little is known of its behavior. It has an electrophoretic mobility indistinguishable from Hgb S; however, the two can be separated by the inability of Hgb D to sickle and a solubility similar to Hgb A.

Hgb E<sup>(9)</sup> is also rare. It can be distinguished by its electrophoretic behavior, migrating to a position between Hgbs C and S. Other studies have indicated it is much like Hgb A.

Hgb G has been reported twice. It has an electrophoretic mobility similar to Hgb F but does not share its resistance to denaturation by alkaline solutions.

Hgb H<sup>(13)</sup> has a more rapid electrophoretic mobility than Hgb A, but other studies show the two to be very much alike.

In addition, other abnormal pigments have been discovered which do not fit into the descriptions of any of the above. Hgbs I and J have been named and probably a host of others are yet to be uncovered.

The so-called hemoglobin traits are those in which the gene for one of the abnormal pigments occurs in combination with the gene for Hgb A. In all traits, except for conditions which will be mentioned later, the A fraction comprises considerably more than half of the total. It is believed that this occurs because the gene for Hgb A is slightly dominant over the genes which produce the abnormal counterparts. All of the hemoglobins inhibit Hgb F, with Hgb A having the strongest inhibitory power. These observations have led to the general belief that all of the hemoglobins, with the exception of Hgb F, represent a set of allelic genes<sup>(2, 15)</sup> which are inherited according to Mendel's laws. The traits represent a heterozygous state.<sup>(10)</sup> The now known traits include AS, AC, AD, AE, AG, and AH.

AS, or sickle cell trait, is the most common and best known of the heterozygous conditions. This genotype is found in ap-

proximately 7% to 9% of American Negroes and produces a condition so mild in degree as to go unnoticed by the patient in most instances. The striking finding is the ability of the red cells to assume the sickled shape when the oxygen tension is lowered. Electrophoresis shows the S hemoglobin to be present in 20% to 46%<sup>(2)</sup> of the total, Hgb A comprising the remaining 54% to 80%. Red cell counts, hemoglobin determinations, corpuscular constants, red cell survival studies<sup>(1)</sup> and fragility studies are within normal limits. A pedigree chart to illustrate the transmission and interaction of the genes for these two hemoglobins is shown in Figure I.

AC, or Hgb C trait, is much less common. It has been reported in only about 2% of American Negroes.<sup>(2)</sup> It is a benign condition producing few symptoms. Hemograms are all within normal limits with the exception of varying numbers of target cells which produce a moderate increased resistance to hypotonic saline. The percent of target cells present, however, does not correlate with the percent of Hgb C. The usual amount of Hgb C is 28% to 44% and electrophoresis easily separates this slow moving pigment from Hgb A in the AC trait.

AD trait is a rare condition which simulates AS in its electrophoretic behavior. However, the red cells containing such a combination fail to sickle.

AE trait has been described extensively in a report from Thailand.<sup>(3)</sup> Approximately 13% of the Thailand population are carriers of this trait. They exhibit no clinical symptomatology and all hematological procedures are within normal limits.

AG has been reported twice and postulated by other investigators. Due to the few cases, little is known about the clinical condition of the patients, but, in general, they present no symptoms.

Similarly, AH carriers are free of symptoms according to the cases reported by Page and his coworkers.<sup>(13)</sup> Electrophoretically they show over 50% Hgb A with the remaining portion having an even faster mobility in the electric field.

If the genes for all of these abnormal hemoglobins prove to be in the same allelic series, the inheritance of any one of the traits can be shown by substituting the letter designating the particular hemoglobin in question for S in Figure I since all of the traits are inherited in like manner.

The clinical syndromes produced by homozygosity for the abnormal hemoglobins are more severe. Of these states, SS, or sickle cell anemia, is the best known and most frequently encountered. Figure I shows how this genetic combination can occur from the mating of two parents who each harbor one gene for Hgb S. One in 600 American Negroes is affected by this disease; there are, as well, frequent cases in Africa, Asia and

Europe. Clinically these people suffer from a severe hemolytic type anemia with joint pains, leg ulcer, sexual immaturity and frequent episodes of severe abdominal pain accompanied by an acceleration of the hemolytic process. Such episodes are termed hemolytic crises. The patients have also been shown to have aplastic crises in which the bone marrow suddenly becomes devoid of nucleated red cells. This condition coupled with the usual fast rate of red cell destruction exhibited by these people can lead to a very serious situation. The sickle cell anemia blood picture reveals a rather severe normocytic, normochromic type anemia, adequate to increased number of platelets, usually a slight leucocytosis with some "shift to the left" of the myeloid series and an occasional normoblast, increased resistance to hypotonic saline solutions, high reticulocyte counts, shortened red cell survival span<sup>(1)</sup> and increased serum bilirubin. Electrophoresis reveals a range of 60% to 99% Hgb S, the rest being Hgb F.

The second most common homozygous state, nonetheless very rare, is CC.<sup>(2)</sup> Its reported incidence is approximately one in 6000 American Negroes. Singer<sup>(19)</sup> and his coworkers in Chicago were the first to report homozygous C disease in four Negro patients. Their findings included a hemolytic process with or without anemia, splenomegaly, numerous target cells and normal levels of Hgb F. Diagnosis was made by electrophoresis. In no case did splenectomy alleviate the hemolytic symptoms. Cases of homozygous C disease have been reported in the Caucasian race though Negroid ancestry has not been ruled out with certainty. Its genetic transmission can be shown schematically by substituting the letter C for S in Figure I.

Homozygous E disease is the only other homozygous condition which has been reported. Its transmission can also be seen by substituting E for S in Figure I. Six patients with this disorder have been encountered in Thailand.<sup>(3)</sup> They exhibit a microcytic, normochromic blood picture, marked number of target cells, increased resistance to hypotonic saline solutions and otherwise normal findings, except for one patient who had 6% Hgb F.

Of the mixed heterozygous hemoglobin diseases there are two known with other possibilities having been reported. The most common combination is that of Hgbs S and C resulting in sickle cell Hgb C disease. Its genetic inheritance is shown in Figure II. It is estimated that these hemoglobins are found together in one in 1500 American Negroes or approximately one-third as frequent as homozygous S. Many cases previously labelled as typical cases of sickle cell anemia have been found to be sickle cell Hgb C disease since the introduction of hemo-

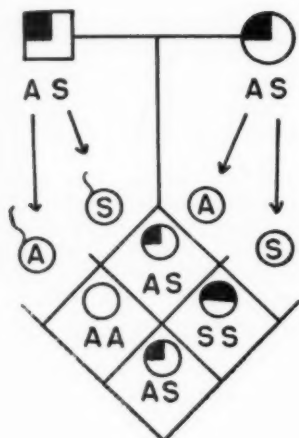


FIGURE I

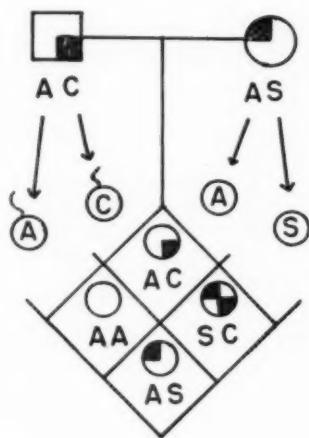


FIGURE II

globin electrophoresis. Clinically, the findings range from almost no symptomatology to a severity resulting in almost complete disability. In the majority of cases, however, the patient is able to lead a much more normal life than one with sickle cell anemia. Crises and leg ulcers are infrequent though they do occur, and splenomegaly is a frequent finding in contrast with sickle cell anemia. Hemolytic exacerbations of the disease are often incurred at the time of delivery in pregnancy. Further complication in patients presenting complaints of hip pain is shown by x-ray to be aseptic necrosis of the hip.

The blood findings are those of a mild normocytic or slightly hypochromic hemolytic anemia. Reticulocyte counts usually are within the high normal range except during periods of crises when all the evidences of a more severe hemolytic process may appear. Target cells are almost always in evidence giving the usual resistance to hypotonic solutions of saline. Red cell survival studies show a survival time ranging between normal and sickle cell anemia, and electrophoresis reveals the two hemoglobin components to be present in approximately equal proportions in most cases.

Insufficient data at present make it difficult to discuss sickle cell Hgb D disease though it seems to produce a severe hemolytic anemia. Summarizing the findings from the few cases reported

there is a normocytic, normochromic or hypochromic anemia, splenomegaly and hepatomegaly, reticulocytosis with nucleated red cells present in the peripheral blood, variable osmotic fragility, sickling and a hemoglobin electrophoretic pattern showing only one component; the two hemoglobins can be separated only by means of the previously mentioned solubility studies.<sup>(6)</sup>

### The Thalassemia Syndromes

Thalassemia is known by almost as many names as are the symptoms it presents. Cooley's Anemia, Mediterranean Anemia and Target Cell Anemia are a few. So many of the cases found have been in people of Mediterranean stock that the term Mediterranean Anemia has been widely used. Neel<sup>(11)</sup> and others<sup>(23)</sup> have postulated that the severe anemia represents a homozygosity and the less severe anemia, heterozygosity, and suggested that the two states be termed Thalassemia Major and Thalassemia Minor respectively. The multiplicity and gradations of clinical features presented by this disease suggest the simultaneous presence of two nonallelic factors.<sup>(23)</sup> Iron deficiency anemias, splenomegalies, lead poisoning, rheumatic fever and certain hepatic diseases have been confused with this disease.

The blood picture in thalassemia reveals a hypochromic, microcytic anemia which is refractory to iron therapy suggesting that the basic defect is an inability to incorporate iron into the heme molecule. There is usually a low mean corpuscular volume (MCV), a low mean corpuscular hemoglobin (MCH) with a low or normal mean corpuscular hemoglobin concentration (MCHC). Target cells vary in number and there is a fair correlation between the number of target cells and the erythrocytic resistance to hypotonic saline solutions. Reticulocytes are high; nucleated red cells and basophilic stippling may be present; and alkaline denaturation studies reveal increased amounts of Hgb F. Electrophoresis shows only types A and F hemoglobins in cases of thalassemia uncomplicated by abnormal hemoglobins.

In addition to Thalassemia Major and Minor, it is now known that other clinical syndromes exist in which genes for at least three of the abnormal hemoglobins occur in combination with the gene for thalassemia. Thalassemia Hgb S disease has been reported by several authors. Sturgeon and his co-workers<sup>(22)</sup> report a subclinical case of Mexican and Italian ancestry who had 70% Hgb S and the remainder a mobility component like Hgb A. The patient's father was diagnosed as having thalassemia minor and his mother was diagnosed as having sickle cell trait with 38% Hgb S.

Powell, Rodarte and Neel<sup>(16)</sup> report a similar condition in a 38-year-old Sicilian who inherited one gene for thalassemia from his mother and one for sickling from his father. They conclude,

as did Sturgeon that the disease resulted from the interaction of two nonallelic genes. Wasserman and his co-workers<sup>(24)</sup> report a hemolytic anemia in a white child as a result of thalassemia and sickling. The symptoms were those of polyarthralgia, fever, jaundice, malaise, pallor, abdominal pain and an enlarged spleen.

The genetic pathogenesis of the sickling trait and thalassemia has been discussed by Silvestroni and Bianco<sup>(17)</sup> and Neel<sup>(11)</sup> who concluded that such a hemoglobin combination is the result of two genes inherited independently which are located on two different chromosomes. How such a combination may occur is shown in Figure III.

### GENETIC TRANSMISSION OF THALASSEMIA AND VARIOUS HEMOGLOBIN COMBINATIONS

FIGURE III

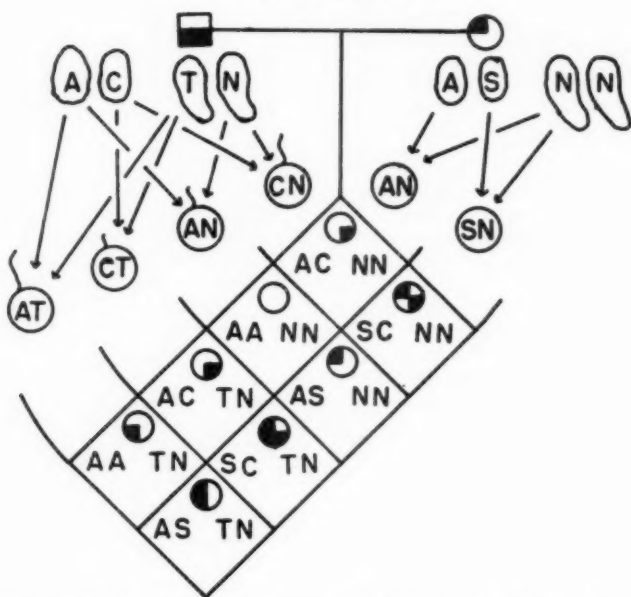


Figure III shows how the genes for thalassemia Hgb C disease are transmitted. This condition has been reported by a few investigators. Singer<sup>(20)</sup> describes a Negro family in which the

blood findings indicated a low MCV and MCH with a normal MCHC, 45% target cells and an electrophoretic pattern revealing 75% Hgb C, the remaining being Hgb A with a small amount of Hgb F in one instance. In all previous electrophoretic patterns of AC trait, Hgb A has comprised over 50% of the total; so it was suggested that in this case, in which it seemed almost certainly the AC trait existed with thalassemia minor, the thalassemia gene enhanced the potency of the abnormal hemoglobin. Zuelzer and Kaplan<sup>(23)</sup> report a case in which the findings were similar except that Hgbs A and C were present in the usual amounts with A having the highest percentage. The symptoms in the patient were too severe to be explained on the basis of either AC trait or thalassemia alone.

Thalassemia Hgb E disease<sup>(3)</sup> is a severe hemolytic disorder presenting symptoms identical with those of thalassemia major. Clinically, jaundice is marked; splenomegaly is striking and hepatomegaly is present in most cases.

Hematologically, these patients have a microcytic, hypochromic anemia with high reticulocyte counts, target cells, spherocytes and nucleated red cells in the peripheral film. Due to the presence of the spherocytes, hemolysis begins in more concentrated saline solutions but is not complete until about 0.12% NaCl. Electrophoretic analysis shows the presence of Hgbs E and F.

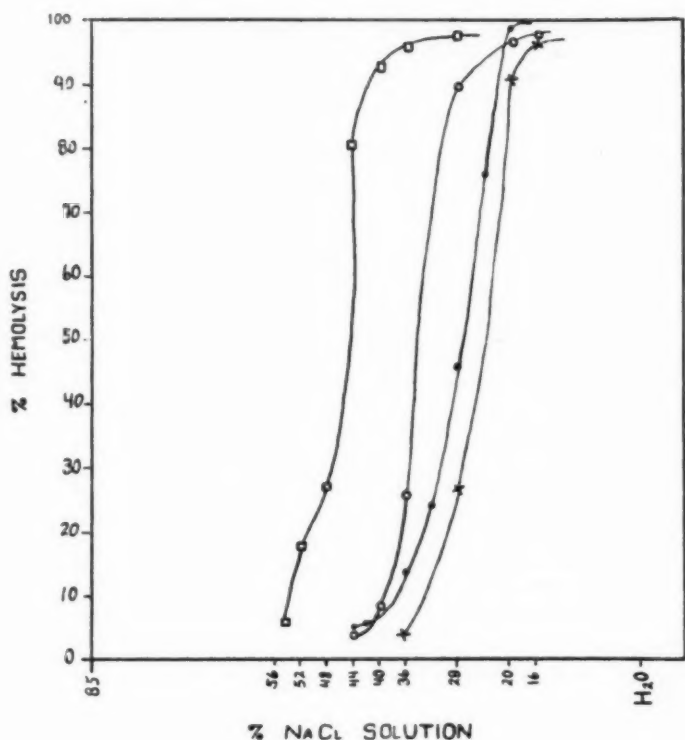
### Materials and Methods

The propositus, a 23-year-old colored male, was first seen at Veterans Administration Hospital in New Orleans. Clinical examinations and hematological laboratory tests were performed on the patient as well as his mother, father, six sisters, one brother, two nieces and two nephews who were available for complete study. A sister, a brother, an aunt and an uncle were not directly available for complete examination and workup, but blood from them was drawn so that some of the laboratory tests could be determined.

The various procedures carried out to supplement clinical workup of the patient and the members of his family consisted of (a) paper electrophoresis, (b) alkaline denaturation, (c) hemogram, (d) osmotic fragility study and (e) red blood cell survival study.

For paper electrophoretic hemoglobin determination the apparatus used was the Spinco Model R cylinder\* with buffer (Spinco Buffer 1), contents pre-packaged to contain 1.84 gm diethyl barbituric acid and 10.3 gm sodium diethyl barbiturate, dissolved in 100 ml distilled water. Buffer pH was 8.6. Approximately 5 ml whole blood were drawn into a tube containing

\* Spinco Model Paper Electrophoresis Apparatus, Spinco Division, Beckman Instruments, Inc., Belmont, California.



- = FRED B, SR. (THALASSEMIA CA HEMOGLOBIN)
- = GAYNELL B. (THALASSEMIA AA HEMOGLOBIN)
- X = CHARLES B. (THALASSEMIA SC HEMOGLOBIN)
- = NORMAL CONTROL

FIGURE IV

potassium and ammonium oxalate anticoagulant, and the hemoglobin solution was prepared by treating the blood as described in the method outlined by Singer et al.<sup>(18)</sup> Hemoglobin in the amount of .01 ml was stripped at the apex on Spinco electrophoretic paper and allowed to run at 22 milliamps Duostat regulated power for 4-5 hours. This technique showed clear

separation of the hemoglobin components. The paper strip was then dried and labelled. Interpretation of the percent of each hemoglobin component was made by use of an automatic photometer—Spinco Analytrol.\*

The alkaline denaturation test was done according to the method of Singer et al.<sup>(18)</sup> using an aliquot of the hemoglobin solution. This is a very sensitive test which detects the presence of alkali resistant component, actually Hgb F. Hemoglobin was pipetted into a tube containing a harsh alkali NaOH (N/12); at the end of one minute the reaction was stopped by the addition of ammonium sulfate (50% saturated) and the mixture was filtered immediately. The filtrate and the untreated hemoglobin solution, diluted 1:200, were read against a water blank in a Beckman spectrophotometer. These data were used to calculate the proportion of alkaline resistant component, Hgb F, in the sample. In our laboratory values of less than 2.0% are considered normal.

The hemogram consisted of hemoglobin, packed cell volume, red blood cell count and percent target cells. Hemoglobin was determined colorimetrically in the Evelyn colorimeter as cyanmethemoglobin; the packed cell volume was determined in the standard Wintrobe tube spun at 3000 rpm for 30 minutes; the red blood cell count was done in duplicate and averaged. The mean corpuscular constants were calculated on the basis of these data. On Wright's stained direct peripheral blood films the number of target cells per thousand red blood cells was counted and expressed as a percent.

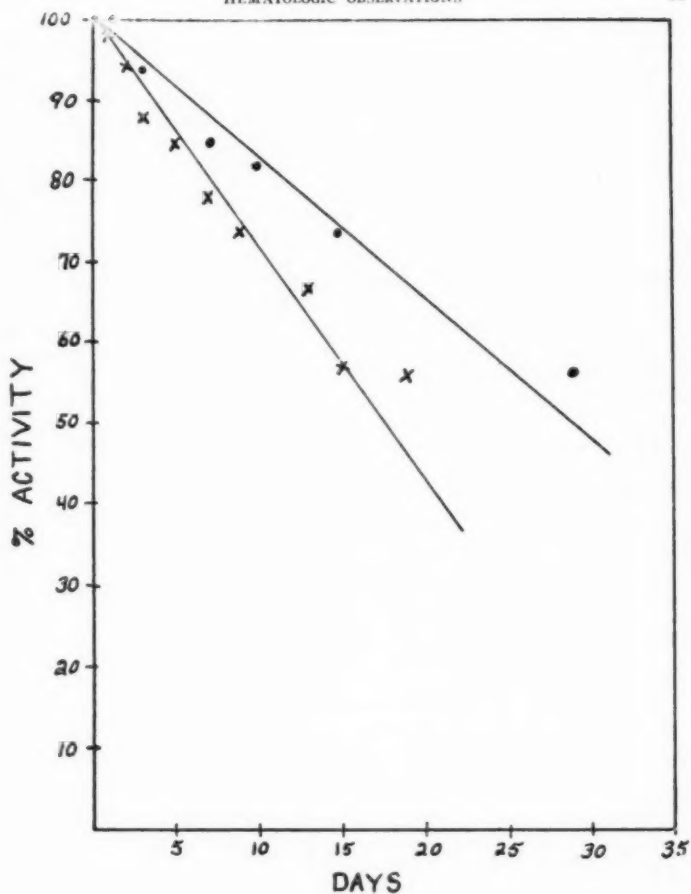
The red blood cell resistance to hemolysis in hypotonic saline solutions was determined by the osmotic fragility test. Sodium chloride solutions of various concentrations ranging from 0.85% to 0.20% and distilled water were used. Into 10 ml of each of the various saline dilutions .02 ml of oxalated whole blood was pipetted, mixed and allowed to stand at room temperature for two hours. The samples were then centrifuged, and the supernates were read in the Evelyn colorimeter. The results were plotted and the curve was compared with the normal curve (0.52%–0.40%) for this laboratory.‡

Erythrocyte survival was determined by means of the sodium chromate ( $\text{Cr}^{51}$ ) technique of tagging red blood cells.<sup>(5)</sup> Under sterile conditions 50 ml of venous blood were drawn and transferred to a siliconized, pyrogen-free bottle containing ACD solution† to which a known concentration (100 mc)  $\text{Cr}^{51}$  had

\* Spinco Model Paper Electrophoresis Apparatus, Spinco Division, Beckman Instruments, Inc., Belmont, California.

‡ After numerous determinations in normal subjects we established the normal osmotic fragility range in our laboratory to be .52%–.40% in contrast to .45%–.30% which is the normal range reported by most hematology texts.

† Special Formula ACD Solution: Dextrose USP 132 mg, Na Citrate USP 250 mg, Citric Acid USP 80 mg, Abbott Laboratories, North Chicago, Illinois.



X = GILBERT B. (SC HEMOGLOBIN)

• = NORMAL

RED BLOOD CELL SURVIVAL STUDIES  
USING CR-51 TECHNIQUE

FIGURE V

been previously added. The ACD solution serves as an anticoagulant and enhances the uptake of the radioactive isotope by the red cells. After careful mixing, the bottle was placed in a water bath at 37° C for 15 minutes. The blood was then reinjected. Six hours later a blood sample was drawn in oxalate anticoagulant, and exactly 2 ml of the blood were placed in a plastic tube. The number of counts per minute of the sample was determined in a Tracerlab Super Scaler scintillation well-type counter. This figure represented radioactivity limited almost entirely to the tagged red blood cells since by this time the radioactivity of the plasma was less than one percent. This initial sample constituted the 100% concentration of activity in the red blood cells. After the sample was counted, it was kept in the refrigerator as a reference standard for all later counts. Specimens of blood were drawn on the first, the second and third day, thereafter at predetermined intervals, and the counts per minute were determined as previously described. At the same time, the original blood sample (6 hours after reinjection) was recounted in order to make correction for physical decay of the radioactive chromium. All counts subsequent to the initial counts were expressed as a percentage of this initial count corrected for physical decay. Samples were drawn and counted until less than 50% activity remained. The percent activity was calculated for each sample and graphed against the day of collection. The day on which the activity curve reached the 50% point indicated the apparent half-life of the red blood cells. No correction for elution of  $\text{Cr}^{51}$  from the cell was made.

### Findings and Conclusions

Except for splenomegaly in the propositus, his father and one sister, the physical examinations of the B. family were essentially negative.

Electrophoretic hemoglobin studies showed the propositus (VI-12) to have an SC hemoglobin pattern with a higher percent of Hgb S (54.5%) than Hgb C (45.5%) and a negligible amount of Hgb F. The hemogram revealed a mild normochromic, normocytic anemia. A peripheral film revealed a marked increase in the number of target cells. The osmotic fragility test showed increased resistance of the red cells to hemolysis (Table 1). The red cell survival study indicated a half-life of 21 days which is significantly shorter than the 30 to 35 days which we have established as the apparent half-life of red cells in normal subjects. Since the clinical syndrome caused by the combination of Hgbs S and C varies so greatly and since the patient showed a higher proportion of Hgb S, it was concluded that he represented sickle cell Hgb C disease.

NAME	NO.	RELATION	AGE	HGB. PATTERN	% HGB.	COMPONENTS	% HGB. F	MCV	MCH	MCHC	OSMOTIC FRAGILITY	TARGET CELLS
BEATRICE B.	8	MOTHER	43	AS	87.1 A	42.9 S	1.7	85	28	33	0.40-0.50	9
FRED B., Sr.	3	FATHER	50	AC	77.5 C	22.5 A	1.8	70	25	35	0.40-0.50	20
WILLIAM B.	4	UNCLE	51	AA	100 A		3.0	PCV 65.0			-----	17
FLORENCE B.B.	5	AUNT	31	AC	80.0 C	20.0 A	1.5	-----	-----	-----	-----	33
RUBY B.B.	7	SISTER	29	AS	51.4 A	48.6 S	1.9	91	31	34	0.40-0.50	28
FRED B., JR.	9	BROTHER	27	AC	58.8 A	41.2 C	0.74	PCV 48.0			-----	36
LOIS B.	10	SISTER	24	AC	55.8 A	44.2 C	0.62	PCV 39.0			-----	25
GILBERT B.	12	PROPRIETOR	23	B <sup>+</sup>	54.5 S	45.5 C	1.6	86	30	35	0.38-0.50	41
GLORIA B.	13	SISTER	22	AA	100 A		0.63	92	29	32	0.38-0.50	5
LETITIA B.	14	SISTER	18	AC	61.8 A	38.2 C	0.56	89	30	34	0.40-0.50	5
ADELE B.	15	SISTER	17	SC	57.7 C	42.3 S	0.75	74	25	33	0.38-0.10-0.50	31
GAYNELL B.	16	SISTER	15	AS	100 A		1.7	74	23	31	0.40-0.50	3
CHARLES B.	17	BROTHER	13	SC	61.3 C	38.7 S	1.4	59	21	35	0.28-0.50	50
BENJAMINETTE B.	18	SISTER	4	AA	100 A		2.3	81	21	31	0.40-0.50	41
EDDIE B.B.	19	SISTER	7	AA	100 A		1.0	81	26	32	0.44-0.50	41
TERRY B.B.	20	NEPHEW	6	AA	100 A		1.4	95	33	35	0.46-0.50	41
CLYDE B.B.	21	NEPHEW	8	AS	57.6 A	42.4 S	1.2	80	28	34	0.40-0.50	41
JARVIS B.	22	NIECE	6	AA	100 A		1.4	86	29	33	0.44-0.50	41

TABLE I

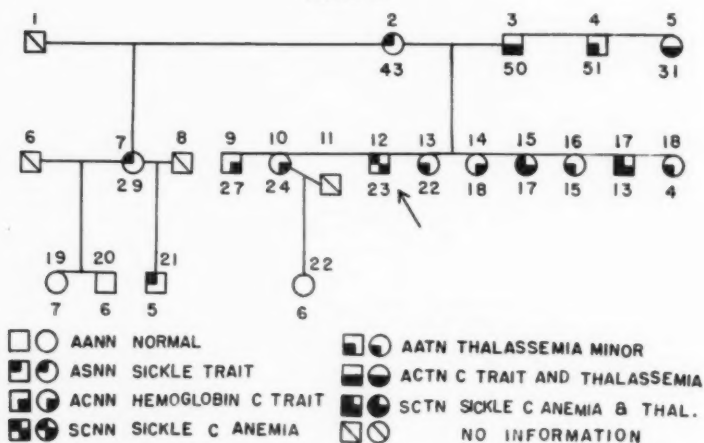
Three sisters (VI-13, 16, 18) and the uncle (VI-4) were homozygous for Hgb A. The uncle and one sister (VI-4, 18) showed increased amounts of fetal hemoglobin; and two sisters VI-16, 18) showed a low MCV, MCH and a normal MCHC. A significant increase in target cells was found in the peripheral blood films of only one sister (VI-13) and the uncle. Osmotic fragility studies on the sisters showed increased resistance of their red cells to hypotonic saline (Table I). Because of the increased number of target cells, the peculiar red cell constants, the decreased osmotic fragility and the presence of abnormal amounts of Hgb F, these members homozygous for Hgb A were diagnosed as thalassemia minor.

Fred Sr., the father (VI-3) and Florence B., the aunt (VI-5) were heterozygous Hgb AC. The presence of an unusually large amount of the abnormal C fraction, similarly seen in a case reported by Singer,<sup>(20)</sup> was the striking feature of their hemoglobin patterns. Hgb F was present in normal amounts. The father revealed a markedly low MCV, MCH with a normal MCHC; the number of target cells was increased in both members; the father also showed a decreased osmotic fragility (Table I). The anemia along with the number of target cells, the inverted ratio of the A and the C hemoglobins and the decreased osmotic fragility made it apparent that Fred Sr. harbored the gene for thalassemia along with the genes for Hgbs A and C. Because Florence B. revealed an even higher inversion of A and C hemoglobin components and a large number of target cells, it is assumed that she also has the entity of thalassemia CA disease. Further studies are needed to prove this diagnosis.

Electrophoretically, Charles B. (VI-17) and Adele B. (VI-15) demonstrated the SC hemoglobin pattern with the proportion

# TRANSMISSION OF THALASSEMIA AND ABNORMAL HEMOGLOBINS IN B. FAMILY

FIGURE VI



of Hgb C being greater than Hgb S. Alkaline denaturation tests showed a negligible amount of Hgb F. Both showed low MCV, MCH with a normal MCHC; Charles revealed a greater degree of microcytosis. Target cells were increased in both, being more marked in Charles. Adele showed a striking decrease in osmotic fragility with hemolysis of her red cells being initiated in a very low NaCl concentration and some cells remaining intact even in distilled water. Charles's fragility curve was similar (Table I). These findings indicate the presence of the gene for thalassemia in addition to the genes for Hgbs S and C. Red cell survival studies remain to be done to further substantiate the diagnosis.

The amount of Hgb F, red cell constants, osmotic fragility and the number of target cells were normal in members VI-19, 20, 21, 22—two nieces and two nephews. The mother (VI-2) and a sister (VI-14) revealed normal hematologic values except for decreased osmotic fragilities and abnormal hemoglobin patterns, the mother AS and the sister AC. The decreased resistance can be explained best by the heterozygous state for an abnormal hemoglobin or possibly a very mild thalassemia minor. In cases VI-7, 9, 10—two sisters and a brother—there was an increase in the number of target cells, all other tests giving values within normal limits; two of these cases (VI-9, 10) presented the electrophoretic pattern of AC and the other (VI-7) that of AS.

### Summary

1. A review of the literature on abnormal hemoglobins is presented.

2. A Negro family is described in which the father and the aunt exhibit the thalassemia CA disease, the uncle thalassemia minor, and the mother uncomplicated sickle cell trait. The siblings represent one case of sickle cell trait, three cases of AC trait, one sickle cell C disease, three of thalassemia minor, and two diagnosed as thalassemia SC disease—a previously undescribed genetic combination. One nephew has sickle cell trait, and two nieces and one nephew are normal.

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## ABSTRACTS

### SUSPECTED CORRELATION BETWEEN BLOOD-GROUP FREQUENCY AND PITUITARY ADENOMAS

Ernst Mayr, Louis K. Diamond, R. Paul Levine, and Margaret Mayr (Harvard University and Children's Hospital, Boston) *Science* 124, 932-3 (1956).

Evidence is noted that the number of cases proving that the blood group genes are not selectively neutral is increasing rapidly. Chi square comparisons of pituitary adenoma with brain tumors yielded the following results. Group O compared with the sum of the other blood groups (A + B + AB):  $\chi^2 = 9.97$ ,  $p = 0.0017$ . Group A compared with the number of the other blood groups (O + B + AB):  $\chi^2 = 18.45$ ,  $p = < 0.0001$ . Since the total number was small, these findings are tentative but do raise some interesting questions. Previously established cases of a correlation between pathological condition and blood group showed that the intestinal tract was involved directly or indirectly. Here an endocrine gland is concerned. What particular attributes of group O should make carriers of group O more and of group A less readily subject to abnormal growth of the chromophobe cells of the pituitary is a complete mystery. The rarity of Group B in the European and North American population is noted yet so far it has not been found to be discriminated against in a single pathological condition. What factor depresses group B to its low frequency is a mystery—some childhood or infectious disease is most suspect.

### ELEVATION OF PLATELETS IN MID-CYCLE: AN INDICATION OF OVULATION

Herman Pepper and Stuart Lindsay (Sequoia Hospital and University of California) *Science*, 124, 189-1 (1956).

A female patient with essential thrombopenic purpura was studied for a prolonged period. It was observed that the platelets consistently reached their highest levels during ovulation as determined by basal temperature graphs. Subsequently twenty-six young women were studied. Following menstruation, the platelets either remained constant or rose gradually during the next two weeks. In mid-cycle, the levels usually rose suddenly and dramatically within 24 hours, reaching the highest point during the entire cycle. Within 24 hours, the platelets returned to their previous levels. This acute rise and fall was as high as 140,000 in some cases. This suggests that platelet levels may be of greater value in establishing the time of ovulation than the basal temperature graphs.

### BACTERIAL HEMAGGLUTINATION AND HEMOLYSIS

Erwin Nater, *Bact. Rev.* 20, 166-88 (1956).

A review (with 156 references) of reactions taking place between red blood cells and bacteria and viruses. These are classified as: (1) direct bacterial hemagglutination; (2) indirect, conditioned, or passive hemagglutination and hemolysis; (3) bacterial hemagglutination and hemolysis of erythrocytes treated with antigen-antibody mixture; (4) bacterial hemagglutination of tannic acid pretreated erythrocytes; (5) bacterial hemagglutination by antibodies against chemically attached protein antigens; (6) red cell linked antigen hemagglutination test; (7) bacterial panagglutination; (8) the bacteriogenic hemagglutination. The interaction between bacteria and their antigens with red blood cells has yielded valuable information on the antigenic composition of various microorganisms and of erythrocytes, and the hemagglutination and hemolytic reactions have been useful as tools for the detection and titration of numerous bacterial antigens and antibodies.

# A COMPARATIVE STUDY OF OXYHEMOGLOBIN AND CYANMETHEMOGLOBIN DETERMINATIONS BY PHOTOMETRIC AND SPECTROPHOTOMETRIC METHODS\*

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In this study, the hemoglobin content of whole blood was compared and evaluated by two standard methods and by using two different instruments: A filter photometer and a spectrophotometer. The investigation was carried out to determine the reproducibility of each method, to discover the variance in values between the two methods, and to determine the correlation in values between the two instruments. The purpose in mind was the selection of a simple, accurate, reproducible method of hemoglobinometry that offered as few problems as possible in instrument calibration so as to enable the average laboratory to periodically check its own instrument.

## I. Introduction

Of all the chemical determinations performed in a clinical laboratory the estimation of hemoglobin is probably done in a greater volume than any other single test. In the light of this, it is discouraging to discover that the results of surveys on the quality of hemoglobinometry show, generally, a very poor degree of accuracy. This is surprising considering that most hospitals use some type of photometric instrument for the determination. The deficiencies may result from inadequacies of the method used, but in a larger part they result from technical errors in the measurement and processing of the blood sample. It is difficult, however, to pinpoint the error of technique unless suitable methods are available for standardization of the reagents and the instrument. Most laboratories rely on precalibration by the instrument maker or on long time-consuming iron or gasometric determinations which have an inherent source of error due to their complexity. As a result of this situation, standard curves are not checked periodically, for validity, as they should be. Two methods for calibration are submitted in the subsequent discourse that are well within the capabilities of any laboratory.

## II. Standardization of Equipment

The possession of an accurate standard or method of standardization is only one of the essentials for accurate hemoglobin measurement. Of equal importance are the instruments, glassware, the training and attitudes of the operators, and the proper use of the standards.

Each factor involved is considered:

*The operator* who performs hemoglobinometry should understand the clinical significance of the test and the necessity for a de-

\* Scientific Products Foundation Hematology 2nd Award. Read before First North American Conference of Medical Laboratory Technologists, Quebec, Canada, June, 1956.

pendable method. He should be well trained in the performance of critical volumetry. He should be familiar with the performance of his instrument so that he is able to identify its behavior.

*All cuvettes* used for hemoglobinometry, even those with manufacturers' marks or specifications, should be matched.

*A. Round Tubes*

1. Clean and dry several dozen cuvettes and make sure they are not flawed, scratched, or etched. Fill each with the same laked blood solution. (Approximately 0.2 ml. of whole blood in 50 ml. of water).
2. Set the photometer at 540  $\mu$  or insert the appropriate green filter. Place one filled cuvette in the instrument well and set the galvanometer beam or meter needle near the middle of the scale. Rotate the tube, noting the slight movement of the beam. At the midpoint of the beam's swing, stop and mark the wall of the cuvette (diamond point) with reference to some mark on the housing of the well.
3. Each of the other cuvettes is now placed in the well and rotated until the reading of the galvanometer beam corresponds with the reading at which the first tube was marked. At this point each succeeding tube is marked in the same way.
4. Precautions should be taken to prevent scratching the cuvettes when using or cleaning them. Test tube racks of wood or rubberized wire are recommended.

*B. Square Cuvettes*

1. Prepare the cuvettes as in Paragraph A1 above.
2. Set the photometer at 540  $\mu$  or insert the proper green filter. Place one filled cuvette in the well of the instrument and set the galvanometer beam or meter needle near the middle of the scale. Note the reading. Replace the cuvette with another and so on until all the cuvettes have been tested and the readings recorded. If inexpensive cuvettes are being used, it should be possible to select from a large lot those with identical readings and reserve them for hemoglobinometry. If expensive cuvettes are being used, it will be necessary to determine the correction factor for each cell and apply this factor in all subsequent determinations and calculations.
3. For convenience and to insure adequate mixing during hemoglobinometry, the blood and the diluent should be mixed in a separate clean, dry tube and transferred to the cuvette at the time it is placed in the instrument.

*Pipettes.*

- A. The 0.02 ml. or 0.025 ml. pipette used to measure the blood should be accurate to  $\pm 1$  per cent. Several supply houses

offer pipettes with a claimed accuracy of this order. However, it would be advisable to calibrate a few such pipettes in order to verify the degree of accuracy. The pipettes should be acid cleaned at least once a week and washed and thoroughly dried between each measurement.

- B. The transfer pipettes used to measure the diluent solution should be of a good order of accuracy and preferably within  $\pm 0.5$  per cent. Some of the commercially available pipettes are well within these limits. (The Bureau of Standards' tolerance on 5 ml. pipettes is  $\pm 0.2$  per cent.) If a burette or automatic pipette is used for this purpose, it should be of the same order of accuracy.

The instrument should be carefully calibrated for photometry according to the instructions provided by the manufacturer. A simplified discussion of calibration of photometric instruments can be found in most laboratory manuals and text books of clinical pathology. For a more detailed discussion of methods of calibration see Drabkin, D. L., *Photometry and Spectrophotometry*, in Glasser, O. (ed), *Medical Physics*, Vol. 2: pp. 1039-1089, Year book Pub., Chicago (1950).

*Standard solutions* should be transferred to matched or calibrated cuvettes that have been carefully cleaned and thoroughly dried. The cuvettes should be firmly stoppered or sealed, if possible. Before use, wipe the surface free of moisture, lint and fingerprints. Ideally, the instrument should be checked by inserting the standard before or after each day's run. Minor unimportant variations occur in most instruments and most operators, after prolonged experience, become familiar with the degree of constancy of their instrument. It is recommended, therefore, that a regular schedule of weekly checking with a standard be instituted. More frequent checking would be within the judgment of the operator.

*The blood* may be taken from a freely bleeding capillary puncture or from a venous sample. The latter must be thoroughly mixed by gently tipping the tube 10 to 20 times before blood is aspirated from it.

*The diluent solution* must be prepared with reagent grade chemicals, should be relatively fresh, and should be free from any growth of yeast or molds. Daily filtration is sometimes indicated.

*Blank.* The diluent solution may be used as a blank. However, the absorption of light by most hemoglobin diluents is negligible so that distilled water may be used as a blank.

*Procedure.* Adapt a method that is suited to the type of instrument used. The manufacturer will generally recommend a procedure.

### III. Experimental

#### A. Hemoglobin

### 1. Oxyhemoglobin Method

#### Reagents:

0.1% Sodium Carbonate

#### Procedure:

To 5 ml. of 0.1% sodium carbonate add exactly 0.025 ml. of whole blood (1-201 fold dilution). Rinse the pipette several times in the diluent and mix well. Allow to stand for 10 minutes for full color development. Transfer to a cuvette and read in the spectrophotometer (Coleman) at 545 mu or in the filter photometer (Leitz) at 550 mu (green filter). Determine the hemoglobin concentration in grams per 100 ml. from a previously prepared calibration chart.

The Coleman spectrophotometer was calibrated by using the Osterberg modification of the iron method of Wong.<sup>19</sup> In this method the iron is released and the proteins separated from hemoglobin by digestion with sulfuric and perchloric acid. The color is developed with potassium thiocyanate and compared with that of a standard iron solution. The hemoglobin concentration is then determined by calculation using the iron content of hemoglobin as 0.335%. The iron standard can be prepared by using iron wire of known percentage or by using ferrous ammonium sulfate (dried to constant weight). This method of calibration is relatively simple and is an accurate means for making periodic checks for calibration validity.

The filter photometer (Leitz) used was a factory calibrated instrument. The method used was the Van Slyke Carbon Monoxide Combining Capacity Method and this was rechecked with standards from the National Research Council.<sup>18</sup>

Hemoglobin values for this method are found in Table #1.

### 2. Cyanmethemoglobin Method

#### Reagents:

Diluent— $\text{NaHCO}_3$ .....	1.0 gm.
KCN .....	50 mg.
$\text{K}_3\text{Fe}(\text{CN})_6$ .....	200 mg.
Distilled water to one liter	

#### Procedure:

To 5 ml. of the diluent add exactly 0.02 ml. of whole blood (1-251 fold dilution). Rinse the pipette several times in the diluent and mix well. Allow to stand for ten minutes for full color development. Transfer to a cuvette and read in the spectrophotometer at 540 mu or in the filter photometer at 550 mu (green filter).

In an effort to standardize hemoglobinometry in the United States, the Division of Medical Sciences of the National Academy of Sciences-National Research Council considered many methods and plans, including the British plan.<sup>1, 2, 3, 4</sup> In respect to simplicity and adaptability, the cyanmethemoglobin method of

the United States Army, which had been subjected to a limited field trial with excellent results, was considered the most suitable for use in the United States and Canada.<sup>6, 7, 8, 9, 10, 11</sup> It was agreed that the choice of a solution of some form of hemoglobin as a standard for hemoglobinometry was both logical and direct. In contrast to a glass standard, it would have the advantage of adaptability to a variety of photometric instruments and cuvettes.

Among the forms of hemoglobin well adapted to photometry, cyanmethemoglobin has outstanding advantages. It has been shown that solutions of the pigment are stable for years when stored at refrigerator temperatures.<sup>12</sup> The absorption band of cyanmethemoglobin in the region of 540 m $\mu$  is broad rather than sharp, so that its solutions are suitable for use in filter type photometers as well as in the narrow band spectrophotometers.<sup>7, 13</sup> Finally, all forms of hemoglobin likely to be found in blood, with the exception of sulfhemoglobin, are quantitatively and stoichiometrically converted to cyanmethemoglobin upon the addition of a single reagent\*.<sup>7</sup>

Specifications were established for the standards and a field trial inaugurated in 1955, but none of the results, to the knowledge of this writer, have as yet been published.

Recommendations for the standard were as follows:

1. There shall be a standard of reference in the form of a preparation of crystalline human hemoglobin by the method of Drabkin.<sup>14, 15, 16</sup> The acceptable criteria for this preparation shall be that a solution containing 1 milliatom of hemoglobin iron per liter shall have a millimolar extinction coefficient of 11.5 at a wave-length of 540 m $\mu$  when measured as cyanmethemoglobin.<sup>7, 13</sup>
2. The iron content of hemoglobin shall be accepted to be 0.335 per cent.<sup>17</sup> This value for iron is the traditional figure used in this country and is in substantial agreement with that adopted by the British. It corresponds with an equivalent weight for hemoglobin of 16,700 per atom of iron and an oxygen capacity of 1.34 ml. per gram of hemoglobin.<sup>17</sup>
3. There shall be a standard for distribution packaged as three separate solutions of approximately 20, 40 and 60

\* Some reluctance has been expressed toward the employment of a standard and of a reagent containing cyanide. The concentration of cyanide in the reagent proposed for use is only 50 mg. of potassium cyanide per liter. Its lethal dose for man approaches four liters and accidental aspiration of a few ml. during pipetting is of no consequence. Most clinical laboratories use for the determination of uric acid a reagent containing 50 gms. of this salt per liter. In view of this, handling of the proposed reagent would seem quite a negligible hazard. However, care should be taken in handling solid KCN. While the concentration of cyanide in the diluent solution is insignificant, the salt itself is highly poisonous and should be handled only by highly responsible people.

mgs. of hemoglobin in the form of cyanmethemoglobin per 100 ml. These three solutions will correspond to approximately 5, 10 and 15 gm. of hemoglobin respectively at a 1-251 fold dilution.

4. The standards will be distributed free of charge to any laboratory that is willing to cooperate in filling out a simple questionnaire and send in periodic reports on the readings of the standards.\*

Hemoglobin values for this method are found in Table #2.

#### IV. Discussion

A summary of the hemoglobin values by the oxyhemoglobin method as presented in Table #1 shows an excellent degree of reproducibility with an average deviation of only  $\pm .17$  gm. using the filter photometer and  $\pm .24$  gm. using the spectrophotometer. On the other hand, values on the average were .33 gms. higher on the narrow band instrument as compared to the filter type (Table #3).

Comparison of cyanmethemoglobin values as presented in

**TABLE I**  
**Hemoglobin Values of Whole Blood as Determined by the Oxyhemoglobin Method, (Gms. per 100 MI.)**

Specimen	SPECTROPHOTOMETER					FILTER PHOTOMETER				
	1	2	3	Average	Deviation	1	2	3	Average	Deviation
1.....	13.3	13.0	13.2	13.2	0.3	13.1	13.1	13.1	13.1	0.0
2.....	15.6	15.6	15.4	15.5	0.2	15.3	15.3	15.3	15.3	0.0
3.....	10.9	10.8	10.6	10.8	0.3	10.5	10.3	10.3	10.4	0.2
4.....	12.2	11.5	11.5	11.7	0.7	11.6	11.2	11.4	11.4	0.4
5.....	13.6	14.2	13.8	13.9	0.6	13.1	14.4	14.0	13.8	1.3
6.....	11.8	11.7	11.8	11.8	0.1	11.2	11.2	11.0	11.1	0.2
7.....	12.2	12.2	12.2	12.2	0.0	11.9	11.9	11.9	11.9	0.0
8.....	14.6	14.4	14.6	14.5	0.2	14.0	14.4	14.0	14.1	0.4
9.....	12.6	12.6	12.6	12.6	0.0	12.2	12.2	12.2	12.2	0.0
10.....	14.2	14.2	14.4	14.3	0.2	14.2	14.2	14.2	14.2	0.0
11.....	13.4	13.2	13.3	13.3	0.2	12.9	12.9	12.6	12.8	0.3
12.....	12.0	12.0	12.0	12.0	0.0	11.7	11.7	11.7	11.7	0.0
13.....	12.9	12.7	12.8	12.8	0.2	12.6	12.6	12.6	12.6	0.0
14.....	10.9	10.8	11.0	10.9	0.2	10.7	10.7	10.7	10.7	0.0
15.....	12.9	12.6	12.9	12.8	0.3	12.8	12.6	12.4	12.6	0.4
16.....	13.1	13.5	13.3	13.3	0.4	13.1	13.2	13.0	13.1	0.2
17.....	13.8	13.3	13.7	13.6	0.5	13.1	13.1	13.0	13.1	0.1
18.....	12.5	12.6	12.7	12.6	0.2	12.2	12.2	12.2	12.2	0.0
19.....	15.7	15.7	15.4	15.6	0.3	15.5	15.5	15.5	15.5	0.0
20.....	12.3	12.5	12.4	12.4	0.2	11.9	12.1	11.7	11.9	0.4
21.....	13.1	13.1	12.8	13.0	0.3	12.5	12.5	12.2	12.4	0.3
22.....	12.6	12.6	12.6	12.6	0.0	12.2	12.2	12.2	12.2	0.0
23.....	10.7	10.8	10.9	10.8	0.2	10.3	10.4	10.2	10.3	0.2
24.....	13.8	13.8	13.8	13.8	0.0	13.4	13.5	13.6	13.5	0.2
25.....	14.2	14.1	14.1	14.1	0.2	13.9	13.9	13.9	13.9	0.0
26.....	13.6	13.6	13.9	13.8	0.3	13.2	13.2	13.2	13.2	0.0
Average Deviation.....					$\pm .24$	Average Deviation.....				$\pm .17$

\* These standards may be procured by writing to R. Keith Cannan, D.Sc., Chairman, Division of Medical Sciences, National Academy of Sciences, National Research Council, Washington, D. C.

average deviation of  $\pm .40$  gm. on the filter photometer and  $\pm .42$  gm. on the spectrophotometer. On the average the values obtained were .18 gm. higher on the spectrophotometer as compared to the filter photometer (Table #3).

**TABLE II**  
**Hemoglobin Values of Whole Blood as Determined by the Cyanmethemoglobin Method. (Gms. per 100 Ml.)**

Specimen	SPECTROPHOTOMETER				FILTER PHOTOMETER						
	1	2	3	Average	Deviation	1	2	3	Average	Deviation	
1.....	13.6	14.0	14.0	13.9	0.4	13.9	13.8	13.7	13.8	0.2	
2.....	13.6	16.3	16.1	16.3	0.5	16.5	16.1	15.9	16.2	0.6	
3.....	11.5	11.5	11.2	11.4	0.3	11.0	10.9	11.1	11.0	0.2	
4.....	12.0	12.0	11.7	11.9	0.3	12.0	12.0	11.5	11.8	0.5	
5.....	14.4	14.6	15.0	14.6	0.6	13.9	14.5	14.5	14.3	0.6	
6.....	12.0	12.0	12.0	12.0	0.0	11.7	11.7	11.8	11.7	0.1	
7.....	12.4	12.7	12.8	12.6	0.4	12.6	12.6	12.4	12.5	0.2	
8.....	15.0	14.8	15.8	15.2	1.0	15.1	15.0	15.1	15.1	0.1	
9.....	13.2	13.2	13.2	13.2	0.0	13.1	13.1	13.0	13.1	0.1	
10.....	15.0	15.0	14.4	14.8	0.6	14.7	14.7	14.0	14.5	0.7	
11.....	14.0	13.6	13.2	13.6	0.8	13.8	13.3	13.3	13.5	0.5	
12.....	12.5	12.5	12.5	12.5	0.0	12.5	12.3	12.1	12.4	0.4	
13.....	14.0	13.2	13.4	13.5	0.8	13.8	13.5	13.2	13.5	0.6	
14.....	11.5	11.0	11.6	11.6	0.4	12.1	12.4	11.5	11.6	0.7	
15.....	13.8	13.6	13.4	13.6	0.4	13.8	13.4	13.0	13.4	0.8	
16.....	13.8	13.8	14.4	14.0	0.6	13.8	13.4	13.8	13.6	0.4	
17.....	14.0	14.0	13.8	13.9	0.2	13.8	14.1	13.4	13.7	0.7	
18.....	13.2	12.7	13.2	13.0	0.5	12.8	12.8	12.8	12.8	0.0	
19.....	15.8	15.8	16.3	15.9	0.5	15.9	15.4	15.9	15.7	0.5	
20.....	13.2	12.8	12.7	12.9	0.5	12.2	12.6	13.2	12.6	1.0	
21.....	12.8	12.8	13.4	13.0	0.6	13.2	13.2	12.6	13.0	0.6	
22.....	13.0	12.8	12.8	12.9	0.2	12.6	12.6	12.6	12.6	0.0	
23.....	11.4	10.8	11.1	11.1	0.6	11.1	10.8	10.8	10.9	0.3	
24.....	14.4	14.4	14.2	14.3	0.2	13.9	14.1	13.9	13.9	0.2	
25.....	14.0	14.2	14.0	14.1	0.2	14.1	14.1	13.9	14.0	0.2	
26.....	14.2	13.8	14.2	14.0	0.4	14.1	13.8	13.9	13.9	0.3	
Average Deviation .....					± .42	Average Deviation .....					± .40

Table #2 also shows a good degree of reproducibility with an

In comparing hemoglobin values obtained by the oxyhemoglobin method against the values obtained by the cyanmethemoglobin method, we find that on the narrow band instrument the cyanmethemoglobin values are on the average .46 gms. higher (Table #3). On the filter photometer the cyanmethemoglobin values are on the average .61 gm. higher than those obtained by the oxyhemoglobin method. We should not expect identical values between methods since variations exist in the conversion of some of the forms of hemoglobin when using different reagents. However, the correlation between the two methods was much better than this writer expected.

## V. Conclusion

The hemoglobin content of whole blood as determined by two standard methods shows that both methods are highly satisfactory for clinical use. The filter photometer and the spectro-

**TABLE III**  
**Hemoglobin Values of Whole Blood—Comparison Between Methods and Between Instruments.**

Specimen	OXYHEMOGLOBIN				CYANMETHEMOGLOBIN				
	Spectro- photo- meter	Filter Photo- meter	Instru. Dev.	Method Dev. Spirntr.	Spectro- photo- meter	Filter Photo- meter	Instru. Dev.	Method Dev. Filtrmr.	
1	13.2	13.1	0.1	0.7	13.9	13.8	0.1	0.7	
2	15.5	15.3	0.2	0.8	16.3	16.2	0.1	0.9	
3	10.8	10.4	0.4	0.6	11.4	11.0	0.4	0.6	
4	11.7	11.4	0.3	0.2	11.9	11.8	0.1	0.4	
5	13.9	13.8	0.1	0.7	14.6	14.3	0.3	0.5	
6	11.8	11.1	0.7	0.2	12.0	11.7	0.3	0.6	
7	12.2	11.9	0.3	0.4	12.6	12.5	0.1	0.6	
8	14.5	14.1	0.4	0.7	15.2	15.1	0.1	1.0	
9	12.6	12.2	0.4	0.6	13.2	13.1	0.1	0.9	
10	14.3	14.2	0.1	0.5	14.8	14.5	0.3	0.3	
11	13.3	12.8	0.5	0.3	13.6	13.5	0.1	0.7	
12	12.0	11.7	0.3	0.5	12.5	12.4	0.1	0.7	
13	12.8	12.6	0.2	0.7	13.5	13.5	0.0	0.9	
14	10.9	10.7	0.2	0.7	11.6	11.6	0.0	0.9	
15	12.8	12.6	0.2	0.8	13.6	13.4	0.2	0.8	
16	13.3	13.1	0.2	0.7	14.0	13.6	0.4	0.5	
17	13.6	13.1	0.5	0.3	13.9	13.7	0.2	0.6	
18	12.6	12.2	0.4	0.4	13.0	12.8	0.2	0.6	
19	15.6	15.5	0.1	0.3	15.9	15.7	0.2	0.2	
20	12.4	11.9	0.5	0.5	12.9	12.6	0.3	0.7	
21	13.0	12.4	0.6	0.0	13.0	13.0	0.0	0.6	
22	12.6	12.2	0.4	0.3	12.9	12.6	0.3	0.4	
23	10.8	10.3	0.5	0.3	11.1	10.9	0.2	0.6	
24	13.8	13.5	0.3	0.5	14.3	13.9	0.4	0.4	
25	14.1	13.9	0.2	0.0	14.1	14.0	0.1	0.1	
26	13.8	13.2	0.6	0.2	14.0	13.9	0.1	0.7	
Average Deviation....			0.33	0.46	Average Deviation....			0.18	0.61

photometer yield equally reproducible results. However, the narrow band instrument gives slightly higher values by both methods. Reproducibility is slightly better in the oxyhemoglobin method, although both show reproducibility within the marginal limits for error. Values for cyanmethemoglobin are slightly higher than those obtained by the oxyhemoglobin method. This may be attributed to the variation in the conversion of some of the forms of hemoglobin when using different reagents.

Each method has its advantages and disadvantages, although minimal ones. The oxyhemoglobin method has the advantage of using an extremely simple and inexpensive diluent, but has the disadvantage of presenting the problem of a more complex and time-consuming method for calibration. The cyanmethemoglobin method has the disadvantage of using a more complex and slightly dangerous reagent, but has the advantage of the availability of a stable, accurate, ready-prepared standard for initial calibration and periodic checking. (This standard can be used for the calibration of other standard methods, such as the oxyhemoglobin method, but not without some loss of accuracy.)

Conversion of the cyanmethemoglobin standards for use with another standard method is carried out in the following manner:

1. Perform on the same sample of blood parallel determinations by the cyanmethemoglobin method and by the method routinely in use.
2. Since the results obtained by the two methods will probably not be identical, provision must be made for converting from one set of values to the other. This is best done by plotting on linear graph paper the results obtained by the dual analyses. One axis of the figure represents hemoglobin concentration by the routine method and the second represents the concentration by the cyanmethemoglobin method. A best smooth curve is then drawn through these points.
3. This graph can be put into general use by entering a value obtained by the routine method on the appropriate axis. This value will intercept the curve at its corresponding point. A projection of this intercept on the cyanmethemoglobin axis gives the hemoglobin concentration of the sample in terms of the standard cyanmethemoglobin solutions.

## VI. Summary

1. The hemoglobin content of 26 different samples of blood was determined in triplicate by two standard methods.
2. The results were determined on a filter photometer and on a narrow band spectrophotometer.
3. Statistical comparison was made between methods and between instruments.

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## ABSTRACTS

### SOME STUDIES ON THE NATURALLY OCCURRING LEUCOCYTE AGGLUTININS

John J. Butler (St. Mary's Hospital, Rochester, N. Y.) *J. Clin. Invest.*, **35**, p 1150-60 (1956).

The purpose of this report is to describe some investigations which attempt to define the nature and incidence of naturally occurring leucocyte agglutinins.

The plasmas of 24 normal donors were tested against the white cells of 7 normal donors. In all, 160 combinations of plasma and white cells were listed and 30 showed agglutination reactions. Two types of cell clumps were noted, the "pure" white clump and the mixed red and white cell clump. Six of the positive tests were of the "pure" white cell variety and these occurred in plasma while cell combinations which were compatible for the ABO blood groups. In 24 of the positive tests the clumps formed were of the mixed cell type; these occurred with combinations which were incompatible. There is no relationship between the leucocyte agglutinins and the ABO blood groups. In that naturally occurring leucocyte agglutinins do exist it would be well in studying a patient's plasma or serum for abnormal leucocyte agglutinins to test it against a panel of leucocytes which are compatible for the ABO blood groups.

### A STUDY OF THE RELATIONSHIP OF CONCENTRATIONS OF PROTHROMBIN, PROCONVERTIN, AND PROACCELERIN TO THREE METHODS FOR MEASURING "PROTHROMBIN TIME"

Lorraine M. Gonyea, Peter Hjort and Paul A. Owren (Department of Medicine, Rikshospitalet, Oslo, Norway) *J. Lab. & Clin. Med.* **48**, 624-33 (1956).

In this paper the terminology of Owren is used. Proconvertin—the stable plasma factor absorbed onto barium sulfate or asbestos filter; its activity is increased with contact with glass, and depressed along with prothrombin, by the administration of Dicumarol and related drugs. Proaccelerin—the labile plasma factor present in fresh or fresh-frozen plasma and which remains in the plasma after adsorption with barium sulfate, asbestos, or the usual adsorbents for prothrombin.

Three one-stage methods for measuring the "prothrombin time" (Quick's method, prothrombin-proconvertin method, and Russell viper venom-cephalin method) were investigated. Dicumarol and related anticoagulants depress the concentration of both prothrombin and proconvertin. The Quick method and the prothrombin-proconvertin method will show this but the Russell viper-venom-cephalin method is insensitive to reductions in proconvertin and should not be used in following anticoagulant therapy.

### OSMOTIC FRAGILITY OF HUMAN BLOOD PLATELETS

J. Gurevitch and D. Nelken (Hebrew University-Hadassah Medical School, Jerusalem) *Blood*, **11**, 924-8 (1956).

This paper presents the behavior of thrombocytes in hypotonic saline: 0.60-0.02 per cent in steps of 0.02 per cent. All glassware was siliconized before use. Blood was drawn into a siliconized syringe containing disodium sequestrene-Triton solution. The blood was quickly centrifuged in a flat-bottomed tube in a horizontal centrifuge for 5-7 minutes at 800 R.P.M. The supernatant plasma was then centrifuged for ten minutes at 2000 R.P.M. to obtain a sediment of platelets. To the platelets thus obtained from 15-20 ml. of blood 1 ml. of normal saline was added, allowed to stand at 6° C with gentle shaking every 10 minutes to make a homogeneous suspension of platelets.

No changes in size occurred at 0.60 per cent although the platelets were more rounded. From 0.60 to 0.44 per cent the platelets were enlarged, apicules longer and fine granules appeared within the cells. Finally at 0.30-0.22 per cent almost all platelets disintegrated.

Also noted was the release of serotonin from the platelets, the amounts increased with the decrease of the NaCl concentration.

## A DEBATE ON METHODOLOGY OF SENSITIVITY: THE TITERED DISK METHOD VERSUS THE GRADIENT PLATE METHOD\*

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With the advent of antibiotics into the field of chemotherapy a whole new field has developed in the routine clinical bacteriological laboratory. Aside from the role of isolation and identification of the etiological agent, the bacteriologist must perform an adequate sensitivity test to determine appropriate antibiotics as an aid to the clinician.

There are so many antibiotics and other chemotherapeutic agents now available for therapeutic treatment that the decision as to which antibiotic to use is becoming increasingly complicated. Clinicians usually treat according to the type of infection and assume what the causative agent might be. Many of the more chronic types of infections, however, (e. g. post operative infection, osteomyelitis, subacute endocarditis, cystitis) are caused by a variety of "less sensitive bacteria" (e. g. *Staphylococcus aureus*, *Escherichia coli*, *Aerobacter aerogenes*), and the establishment of the etiological agent is of the utmost importance. The infections caused by these "less sensitive bacteria" as contrasted to infections caused by pneumococci, Group A beta streptococci, gonococci (e. g. always sensitive to penicillin) often do not respond to recommended therapeutic agents. Therefore, the laboratory serves as an aid in performance of sensitivity tests. The necessity for an adequate test is of prime importance, and this test must be relatively accurate, fast, inexpensive, and reproducible.

There exists quite a bit of controversy as to the best type of sensitivity test to perform in the clinical laboratory, and this controversy is based primarily on which test will give the clinician the most accurate results in the least amount of time possible. In general, there are two types of sensitivity testing procedures performed in the clinical laboratories today: (1) the serial dilution method which is a quantitative one giving an end point indicating the least concentration of the drug which inhibits growth and (2) the agar diffusion method which involves diffusion of the active agent into a solidified medium from a reservoir of the antibiotic. The latter is at most a semi-quantitative or a qualitative test.

### A REVIEW OF METHODS

**Serial dilution method.** The serial dilution method consists in the use of a series of tubes of a liquid culture which receive varying amounts of the drug in rather closely graded steps and then

\* Scientific Products Foundation Bacteriology First Award. Read before First North American Conference of Medical Laboratory Technologists, Quebec, Canada, 1956.

inoculated with a number of organisms. There are some major factors influencing the results of the serial dilution test that Ericsson and Tunefall (1954) have pointed out, such as the composition of the medium, size of the inoculum, and incubation time. The incubation time in the serial dilution test, when prolonged, may cause inactivation of the active substance, especially the bacteriostatic agents such as aureomycin, achromycin, terramycin, and chloromycetin. The deterioration of these drugs seems to cause more difficulty in the reading of the tests. To avoid deterioration, the tests involving these different antibiotics should be read at optimum incubation times and tested in optimum mediums. This would give comparable results for all agents. But this is not practical for routine work.

It is generally agreed that the serial dilution method is the most accurate method with evaluations carried on by Ericsson (Et al., 1954), Gunnison, and Jawetz (1953), Howe (1950), Hoyt (1952), and Waisbren (1951): but its use is limited more to the research type of laboratory. In performing the test, a large amount of time, equipment, material, and effort are consumed. When there is only one person doing all of the bacteriological tests, the use of the serial dilution test is out of the question. However, more rapid modifications of the tube tests have been reported by Schneierson (1954) and Schwanz (1954). These were not investigated at this time.

**Agar plate diffusion method.** The theory behind the diffusion method of assaying antibiotics is based on the fact that there is a diffusion of the antibiotic into the agar from the reservoir of antibiotic, such as an assay cylinder, or paper disk. These latter types of diffusions can be considered as the diffusion of a solute in a colloid and therefore, following Fick's formula (Miyamura, 1953), a quantitative estimation of the amount of antibiotic that the organism is sensitive to can be calculated. This is dependent upon the volume and concentration of antibiotic, the zone of inhibition and the unit diffusion into the agar. There is a steep logarithmic function involved in this lateral type of diffusion, and a wide range of error might be involved by close quantitative calculation. The factors of error, such as the type of medium used, as Waisbren (et al., 1951) has pointed out, the depth of the agar, the diffusability and stability of the antibiotic through the agar, and the moisture of the agar surface play a big role in affecting the final sensitivity results.

Because of the varying zones of inhibition which have been obtained in running triplicate tests on a single bacterium, there may be a variation of as much as 25 percent in agreement (Spaulding and Anderson, 1951). For this reason, many laboratories will report only qualitative results (e. g. sensitive or resistant), regardless of the size of the zone of inhibition by the antibiotic. Bondi and his associates (1947) however, in their original method, reported their results in a semi-quantitative manner with four gradations. An editorial, A. M.

A., Editorial on Disc Method, 1951, pointed out that "the antibiotic that produces the largest zone of inhibition of bacterial growth on the culture plate is not necessarily the one to which the test organism is most sensitive."

The plate diffusion method does offer the following advantages over the serial dilution method: (1) several antibiotics can be tested on a single plate, (2) the medium used may be selected to meet particular growth requirements from the stock of plate media kept on hand, (3) there is an ease and economy of both effort and materials, (4) amount of chemotherapeutic agents contained in the disks may be chosen so as to make sensitivity zones correspond to therapeutic serum levels, and (5) the incubation time may be arbitrarily chosen and identical for all agents.

**Gradient plate method.** A new method, the gradient plate method, was introduced in 1952 by Szybalski as a method for genetic study, and Braude (1954) suggested its use as a routine clinical determination of bacterial sensitivities. The gradient plate used (Figure 1) appeared to have unique possibilities as a routine clinical sensitivity test because it is a quantitative test and more than one organism could be streaked on a plate. Because Szybalski recognized the failings of the serial dilution method and the use of the agar plate in contact with a reservoir antibiotic he worked out the simple gradual proportional increase of the antibiotic concentration along one horizontal axis—the gradient. This mechanism (Figure 1) shows how there is an upward diffusion of the antibiotic when placed in the bottom layer of agar. In this diffusion, there is a dilution proportional to the ratio of the thickness of the agar layers. There is a uniform linear concentration gradient during subsequent incubation. The reverse in layers also holds true. Actual measurement of growth is determined by the formula  $D_1/D_2 \times$  initial concentration of antibiotic, where  $D_1$  is the distance of the point of growth—inhibition from the beginning of the gradient and  $D_2$  is the diameter of the plate.

The debate begins against this background of information about sensitivity tests. The investigators in this debate represent two hospital laboratories, each serving approximately 300 to 400 beds. The titered disk method (agar plate diffusion) is used routinely by Investigator I for all sensitivity tests, while the gradient plate method is used exclusively by Investigator II, having changed to this method after comparative sensitivity studies. The two investigators were interested in determining the method of sensitivity study that would be the most accurate and yet practical for their particular bacteriology laboratories. They, therefore, set up a series of experiments to evaluate the titered disk and the gradient plate method using the same test organisms under similar conditions. Modifications of the tests were studied within the individual laboratories and a comparative evaluation

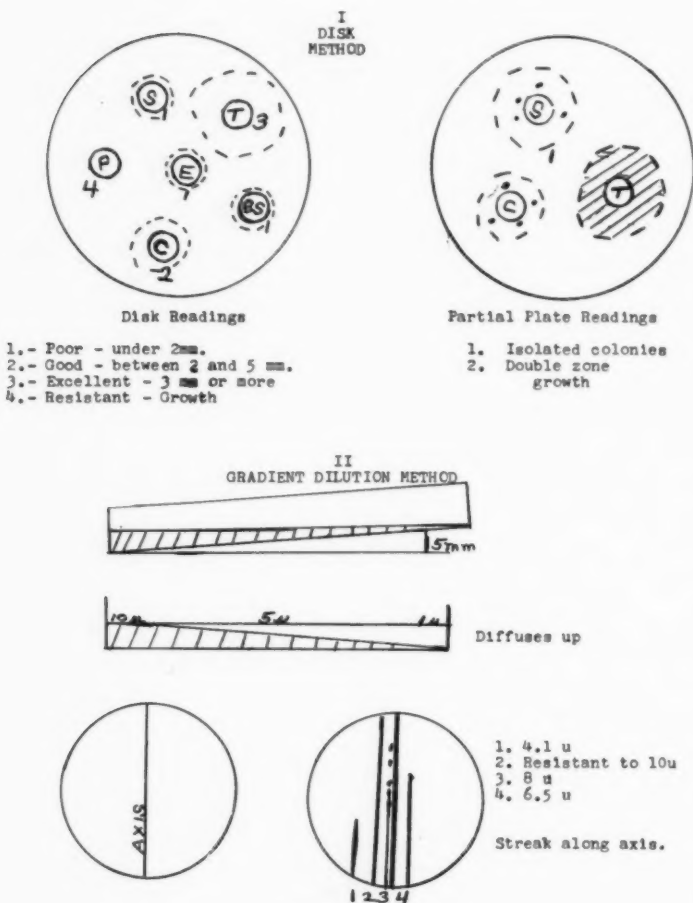


FIGURE I

THE DISK AND GRADIENT PLATE METHODS

of the two methods was made.

#### PERFORMANCE OF THE IN VITRO TESTS

**Test organisms and culture media.** Thirty-three test organisms were used in the comparative studies between Laboratories I and II and were originally isolated from cultures of blood, urine, stools, upper respiratory secretions, and abscesses, and included

strains of *Escherichia Coli*, *Aerobacter aerogenes*, enterococci, *Staphylococcus albus* and *Staphylococcus aureus*. The test organisms were some that had been in a previous study of the gradient plate method in Laboratory II and the original results were used for a comparative evaluation in this study. The organisms were kept in stock culture on plain agar slants submerged in sterile mineral oil and the tests were performed using eighteen hour broth culture transplants. The agar media used in this study was trypticase soy agar (Baltimore Biological Laboratory) with and without five percent human blood added.

**Antibiotics.** The five antibiotics which were used consisted of tetracycline, chlormycetin, erythromycin, streptomycin (dihydrostreptomycin) and penicillin. The working concentrations of these antibiotics are to be found in Table I. Two forms of antibiotics

TABLE I  
Key to Antibiotic Concentrations Used in the Disk-Gradient Tests

Code Letter	Antibiotic	CONCENTRATION						
		Gradient	Titrated Disk					
			Bacto		Sensi		Desl	Multi
			High	Low	High	Low		
C	Chlormycetin	10 mcg	10 mcg	5 mcg	30 mcg	5 mcg	10 mcg	10 mcg
E	Erythromycin	5 mcg	5 mcg	2 mcg	15 mcg	2 mcg	10 mcg	10 mcg
P	Penicillin	2 mcg	5 unit	2 unit	10 unit	2 unit	1.5 unit	1.5 unit
S	Streptomycin	10 mcg	10 mcg	2 mcg	50 mcg	10 mcg	10 mcg	10 mcg
T	Tetracycline	10 mcg	10 mcg	5 mcg	30 mcg	5 mcg	10 mcg	10 mcg

were used for the tests: (1) commercially prepared disks containing known quantities of antibiotics and (2) water soluble powder forms obtained from the respective hospital pharmacies.

Four commercial disks were used in the disk comparative studies: (1) Desi-Discs, (National Bio-Disc Co.) (2) Sensi-Discs, (Baltimore Biological Laboratory) (3) Multidisks, (Consolidated Laboratories) and (4) Bacto Disks, (Difco Laboratories).

The water soluble forms were diluted to 1000 micrograms per cubic centimeter with exception of penicillin (1000 units). Sterile water and saline were used in the preparation of the stock solutions. They were then distributed into test tubes and stored at minus 8° centigrade with the potency of each remaining stable for at least six months. The final dilutions of 10 micrograms, 5 micrograms and 2 units were made from these stocks.

**Tests for sensitivity.** (1) The disk diffusion method was performed by streaking either a 90 millimeter or 140 millimeter petri plate with a saturated swab of the appropriately diluted 18 hour broth culture organism. Laboratory I diluted the culture of the test organism I to 500 in sterile saline and Laboratory II used the undiluted culture of the test organism. Filter paper disks

containing appropriate concentrations of the antibiotic were placed on the petri plates with flamed forceps, incubated at 37° centigrade and were read in 18 to 24 hours.

(2) In the gradient plate method three or four of the 18 hour broth culture organisms were streaked along the axis of the gradient under the same conditions as stated for the disk method. The antibiotics previously were added to 100 cubic centimeters of melted agar (48° centigrade) to give appropriate final dilutions. The agar was then poured into seven or eight 90 millimeter petri plates for each antibiotic tested and slanted on a six millimeter board as shown in Figure 1. The first layer of agar was allowed to harden, the axis was marked, and an equal amount of plain agar containing no antibiotic was poured as a second layer. After final hardening, the plates were held in the icebox overnight before streaking.

**Measurement and reporting of results.** The strictly qualitative method of reporting the agar plate disk diffusion method was followed in Laboratory I, thus recording only the presence or absence of growth around the disks. However, Laboratory II recorded the disk results in a semi-quantitative method as shown in Figure 1. The quantitative measurement of growth on the gradient plate was recorded according to the formula given earlier (Braude, 1954). When there was partial growth, measurement was taken from the last point of visible growth.

Correlation in the results of the disk studies between Laboratories I and II were made as qualitative ones when comparing results at the same antibiotic dilutions. A satisfactory agreement between the gradient plate sensitivity tests was considered a correlation within less than 30 percent of the maximum antibiotic concentration.

#### A CASE FOR THE TITERED DISK

**Results of the Disk Test.** The results of the studies can be seen in Tables II through VI. Investigator I compared the commercial Sensi-Discs and the Bacto Disks, using the High-Low concentrations found in Table I and reading the results as a qualitative test. This investigator noted an agreement of 97.6 percent. Investigator II compared Multidisks and Desi-Disks, using a semi-quantitative reading technique of measured zones of inhibition, and noted an agreement of 91.7 percent. The four commercial disks were compared between the two laboratories (Table IV) and a 90.8 percent agreement was noted. This closely compared with the percent of agreement found between the two disks studied in Laboratory II using a semi-quantitative method of measuring the zones of inhibited growth around the disks. This rather poor agreement of the results obtained comparing qualitative with semi-quantitative, and semi-quantitative with semi-quantitative would indicate the probable benefit of qualitative inter-

**TABLE II**  
**Comparison of Two Different Commercial Disks in Laboratory I**  
**(33 Test Organisms)**

Antibiotic	Total Tests	Agreements No. of Tests	Disagreements No. of Tests
P	33	31	2
E	33	33	0
T	33	32	1
C	33	33	0
S	33	32	1
Totals	165	161	4
Percent	100.0	97.6	2.4

**TABLE III**  
**Comparison of Two Different Commercial Disks in Laboratory II**

Antibiotic	Total Tests	Agreements No. of Tests	Disagreements		
			No. Tests	MMS*	DMS†
P	48	39	9	9	0
E	48	46	2	0	2
T	48	47	1	1	0
Totals	144	132	12		
Percent	100.0	91.7	8.3		

\* Multidisk more sensitive.

† Desi-disks more sensitive.

**TABLE IV**  
**Comparison of Disk Tests Between Laboratories I and II**  
**(31 Test Organisms)**

Antibiotic	Total Tests	Agreements No. of Tests	Disagreements		
			No. Tests	LIMS*	LIIMS†
P	13	8	5	5	0
E	13	12	1	1	0
T	31	31	0	0	0
C	31	29	2	2	0
S	31	28	3	3	0
Totals	119	108	11		
Percent	100.0	90.8	9.2		

\* Laboratory I more sensitive.

† Laboratory II more sensitive.

pretation of the titrated disk method. Those who favor this method of disk reading emphasize the many variables that enter into a test of this nature. Independent of the sensitivity of the bacteria being tested, there are many other factors that play an important role in the determination of the size of the zones of inhibition. These factors have been presented in the section introducing the agar diffusion method. This does not imply that there is no relationship between the size of the zones of inhibition and the

**TABLE V**  
**Comparison of Gradient Tests Between Laboratories I and II**  
**(32 Test Organisms)**

Antibiotic	Total Tests	Agreements No. of Tests	Disagreements		
			No. Tests	LIMS*	LIIMS†
P.....	14	9	5	5	0
E.....	14	13	1	1	0
T.....	32	31	1	0	1
S.....	32	26	6	4	2
C.....	32	32	0	0	0
Totals.....	124	111	13	10	3
Percent.....	100.0	89.6	10.4		

\* Laboratory I more sensitive.

† Laboratory II more sensitive.

**TABLE VI**  
**Comparison of Gradient Tests in Laboratory II**  
**(32 Test Organisms)**

Antibiotic	Total Tests	Agreements No. of Tests	Disagreements No. of Tests
P.....	14	14	0
E.....	14	14	0
T.....	32	30	2
S.....	32	27	5
C.....	32	31	1
Totals.....	124	116	8
Percent.....	100	94	6

sensitivity, but that it is not the most dependable method of interpretation.

**Discussion:** The titered disk is a series of two or more disks saturated with different concentrations of antibiotics. These different concentrations should be representative of the effective level *in vivo*. If an organism is highly sensitive to an antibiotic a very low dilution of that antibiotic should inhibit the growth of the organism. The titered disk is so prepared. The High-Low disks will indicate the range of the sensitivity of the organism. Some disks on the market have a titer in excess of the serum level which may be obtained *in vivo*. These higher titered disks are of value in urinary tract studies where higher levels can be obtained in the urine (Table VII). However, in all other instances, to report as sensitive to antibiotic levels which cannot be obtained in the serum, is possibly to give misleading information to the clinician. Consequently, it is the responsibility of the bacteriologist to be informed of the serum levels that, under favorable conditions, can be obtained *in vivo*. These levels have been reported in current literature and have been collected in Table VII.

Investigator I desiring to establish the case for the titered disk

TABLE VII  
Therapeutic Urine and Serum Antibiotic Levels

Antibiotic	Blood Serum Level	Urine Level
Penicillin	0.01-2 units Upper level 10 units	
Erythromycin	0.2-3 micrograms Upper level—8 micrograms	50-80% Excreted
Streptomycin	4-20 micrograms Upper level—40 micrograms	100-400 micrograms
Chloromycetin	10-30 micrograms	
Oxytetracycline (Terramycin-Phizer)	0.5-8 micrograms	
Tetracycline (Achromycin-Lederle)	1.25-5 micrograms	30-125 micrograms
Chlortetracycline (Aeromycin)	2-4 micrograms	32-256 micrograms
Bacitracin	1-10 units	
Polymyxin B	1-8 micrograms	2-48 micrograms
(Aerosporin)	10-80 units	20-480 units
Neomycin	4-10 units	

method feels that the 97.6 percent agreement between the two sets of disks studied in Laboratory I indicated a good degree of reproducibility and uniformity.

A good method of antibiotic sensitivity testing would have to be one that employed a method of antibiotic assay that would guarantee the stability of the antibiotic regardless of the source. Lind, Branch and Power<sup>1</sup> report that disks manufactured by one of the company's disks studied in this report remained stable for a period of two years when kept tightly stoppered at 2-10° centigrade.

The number of disks used will vary from one sensitivity to another depending on the organism being studied. However, an average cost of one and a half cents per disk plus a small additional cost for the agar used brings the total cost of the titered disk diffusion method to where the test can be used as a routine procedure.

It is difficult to compare the *in vitro* sensitivity data with the *in vivo* response. *In vivo* there are other sets of conditions in which the number of infecting organisms may vary markedly and contrast greatly with the conditions used in the *in vitro* test. It is with this in mind that Investigator I wishes to point out that, at best, the sensitivity study is a qualitative test where the bacteriology laboratory can only present the doctor with a guide to distinguish sensitive from non-sensitive organisms.

The experience of Investigator I with the gradient plate method is limited to the study of the thirty-three test organisms. With instructions from Investigator II, the experiment was carried out with ease.

The most difficult part of setting up the experiment was met in

<sup>1</sup> Difco No. 146, November, 1955, *Microbial Sensitivity Testing with Bacto Sensitivity Disks*, pp. 16.

preparing the dilutions of the antibiotics. This is a mathematical problem in dilution.

The most time consuming procedure in this method of sensitivity study is found in the preparation of the gradient plate. The antibiotics, once diluted, are frozen and must be stored in a deep freeze unit. This requires a lot of space in the deep freeze, because the diluted antibiotics are necessarily prepared in small lots. Large numbers of plates are also required because only one antibiotic can be tested per plate, and to be practical, a large volume is prepared at one time. This also necessitates a large refrigerated storage space for the plates.

Square plates would show the gradient technique to best advantage, but these are not available. These would make the gradient axis easier to establish and the streaks of bacteria being tested more uniform in length. As it is now used, the lengths of the streaks vary and the measurement of the bacterial growth is more complicated the further the streak is from the line of the axis in the center of the plate.

The gradient plate is not as stable as the titered disk and may be kept for use for only about one or two weeks. This fact coupled with the involved time consuming process of preparing the gradient plates would not be favorable factors for its adoption in a laboratory that does not perform an average of more than two or three sensitivities a day.

The highest concentration of the antibiotics now used in the gradient plate as shown in Table VII are not representative of the higher levels that can be obtained *in vivo* in urinary tract infections. There are instances of resistance to antibiotics at lower levels when higher concentrations might show a sensitivity indicating these antibiotics as the most preferable therapeutic.

Braude (1954) in his studies found discrepancies in the use of erythromycin in the gradient plate. The basis for this was not discovered, but it was proposed that a possible reason was that this antibiotic diffused so rapidly throughout the medium that the gradient was lost. Penicillin, also, showed a few discrepancies where penicillinase producing organisms were inhibited by low concentrations of penicillin on the gradient plate.

**Conclusions.** Properly used and properly interpreted, the titered disk is reproducible, stable, inexpensive and is a practical qualitative method of studying bacterial sensitivity. Because of numerous conditions involved in determining both the *in vitro* sensitivity and the *in vivo* response there seems little practical necessity for performing a test more accurate than a qualitative estimate of the sensitivity of the bacteria being studied. The growth media of the bacteria as they are reproduced *in vitro* are at best only a good foster home and cannot more than resemble the conditions of their *in vivo* habitat. The body fluids and the tissues present a set of living conditions that

are not reproduced *in vitro*. The individuals themselves have different defense mechanisms which go into action with the antibiotic given for treatment. The serum and urine levels of the antibiotic are completely individual. These factors help to make clearer the limitations of the sensitivity test and emphasize the qualitative nature of the test.

#### A CASE FOR THE GRADIENT PLATE

**Results of the Gradient tests.** The results of the gradient tests are noted in Tables V and VI with the addition that Table VI also shows the comparison within Laboratory II of the gradient plate method. The agreement of the gradient plates between Laboratories I and II was 89.6 percent, noting that high in the disagreements were penicillin and streptomycin. Laboratory I gave more sensitive results with both of these antibiotics. In contrast, the reproducibility within Laboratory II of the gradient plate was 94 percent with only streptomycin high in disagreement.

**Discussion.** There has been a demand on the laboratory by clinicians, since sensitivity tests were first started, to determine which antibiotic is the most effective *in vitro*. This knowledge can only be obtained by finding out the exact quantitative sensitivity level. The gradient plate method offers to the routine clinical laboratory an easily performed quantitative sensitivity test. The advantage over the disk diffusion method is found in a very gradual proportional increase of the concentration of the antibiotic along the horizontal axis of the gradient. With the disk method, the concentration of the lateral diffusion, which is in respect a gradient, represents a steep logarithmic function with the area being narrow between the desired concentrations needed to distinguish clinically sensitive from non-sensitive bacteria. The pitfalls of semi-quantitative reporting of sensitivity zones using a one disk concentration have already been stated.

In reality, the titered disk method used by Laboratory I is a semi-quantitative test when finding the relative end-point range of the organism by qualitative readings of different disk concentrations. This can be compared to the serial tube dilution, with the same results being obtained. Whereas Laboratory I must use two to three disks in the titered disk method, Laboratory II has the gradual increase on one plate with an exact end point established. The antibiotic range to be selected has already been discussed and its importance can't be emphasized enough. It is possible to have a graded antibiotic range corresponding to serum levels on the smaller petri plates (90 millimeter) and one corresponding to urine levels on the larger petri plates (140 millimeter).

A good sensitivity test must be reproducible in all laboratories and not just within one laboratory. Investigator II has been using the gradient plate sensitivity test in routine work for almost a year and wanted to see if the test could be adapted with repro-

ducibility and ease of performance in another laboratory.

It is to be noted that the agreement between Laboratories I and II was 5 percent less than that within Laboratory II. However, in analyzing the reason for disagreement between the laboratories, some interesting points were brought out. For example, penicillin gave a high percentage of disagreement. Some of the organisms that were resistant to 2 units of penicillin in Laboratory II were sensitive to less than 0.2 units in Laboratory I. One of the differences in performing the comparative tests between the laboratories was the dilution factor of the broth culture test organisms. Investigator I diluted the broth culture 1:500 whereas Investigator II tested them undiluted. Desiring to discover whether or not the dilutions were the direct cause of disagreement between the laboratories, Investigator II set up some additional tests comparing the organisms that were in disagreement. Diluted and undiluted broth cultures were streaked on one gradient plate containing penicillin. In every case, the undiluted organism giving a resistance to over 2 units of penicillin gave a sensitivity increase of over 1.5 units in the 1:500 dilution. When testing on gradient plates containing the other antibiotics, the difference in the dilution factor did not cause a significant change in final results. The conclusion can be made, then, that the two laboratories were not in disagreement on their comparative penicillin studies. If the error were corrected, there would be an agreement between Laboratories I and II of 93.8 percent. This figure compares favorably with the reproducibility within Laboratory II of 94 percent. Investigator II in support of the case for the gradient plate finds it an outstanding fact that the first time Investigator I ran the set of gradient tests in a completely different laboratory with separate equipment an agreement of these like percentages was obtained. This indicates the ease of reproducibility of the test.

There were differences of 4 to 5 micrograms in the comparative readings on the plates containing streptomycin, which leads one to assume that a mechanical difficulty might have been present. The mechanical difficulties in the preparation of the gradient must be taken into consideration when trying to obtain reproducible results. These difficulties might be due to incorrect measurement of the chemotherapeutic agent when making dilutions, preparation and pouring of the two layers of agar, or improper marking of the axis for each gradient. The mechanical variation was set at three-tenths of the gradient axis of the petri plate. This was determined by streaking the test organisms on plates prepared at different times, with different lots of antibiotics. The reproducible results are found in Table VIII. After varying the amounts of the maximum antibiotic concentration on the gradient, the most workable concentration was found to be 10 micrograms or under for the 90 millimeter petri plate. The range of error

**TABLE VIII**  
**Mechanical Variation in Reading of Gradient Plates**

Test Organism	ANTIBIOTIC SENSITIVITY READINGS (Micrograms/cc) <sup>a</sup>					
	T	AC	S	C	E	P
<i>E. coli</i> (S-226).....	5, 2, 3					
<i>E. coli</i> (S-229).....	5, 4, 4		7, 9			
<i>E. coli</i> (S-219).....			6, 5, 7		S, S†	
<i>Staph. aureus</i> (S-121).....	S, 1, S, S	S, S	7, 4	7, 5, 7, 8		
<i>Staph. aureus</i> (S-116).....	S, S	S, S	8, 7		S, S	S, S
<i>Staph. aureus</i> (S-119).....	S, S	S, S	3, 5	6, 7	S, S	

<sup>a</sup> Exception is penicillin (units/cc).

† S represents sensitive to less than 1 unit or 1 microgram/cc.

would increase when antibiotic concentrations over this are used.

One of the reasons that Investigator I had in changing from the paper disk to the gradient plate method was that some discrepancies were found in an earlier study on commercial disks. Investigator I states that the gradient plate is not as stable as the disk, which is true in that some plates (e.g. penicillin) can be kept under refrigeration for only one week. Yet, Investigator II found definite discrepancies in this present study between a few commercial disks of penicillin. On the same test organism, at the same dilution, one disk read resistant to 1.5 units of penicillin and another gave a good zone of sensitivity. Investigator II definitely found a deterioration of penicillin with age when diluted in the gradient plate, so by the same token, disks containing penicillin, when not properly stored may also deteriorate, resulting in false resistant disk zones. This could misguide a clinician in therapeutic treatment.

Sensitivity zones around 5 micrograms of erythromycin disks were noted with some of the enteric Gram negative rods. Yet on the gradient plate tests the same organisms were resistant to 5 micrograms of erythromycin, the dilution of the test organisms being the same. Erythromycin is generally known to be ineffective against enteric Gram negative rods in vitro, yet a sensitive disk reading was obtained. These results indicate a false zone around the disk and an erroneous report would be made.

Investigator I pointed out the difficulties in the mechanics of the gradient test. The preparation of the stock antibiotic solutions only offers a problem once every six months, as these solutions can easily be prepared in one afternoon and then stored and kept stable for that long a period in a deep freeze. The necessity for a large space in preparation of the plates and for their storage is recognized and for this reason, the performance of the gradient test is limited to larger laboratories.

The actual cost of the antibiotics used for each test plate is less than 0.5 cents as compared to 1.5 cents for the commercial disks. When four test organisms are streaked on a plate the cost is cut

even more. A real advantage for the gradient plate is found in the laboratories performing more than four sensitivity tests a day. The time consumed in performance of the gradient test is cut in half as compared to the performance of the titered disk method. This factor is of prime importance in a busy laboratory.

Because of the axis type of streaking required on the gradient plate, a round conventional type of petri plate has its drawbacks. A square plate would be much more beneficial; but yet, even though the lengths of the streaks of the organisms vary the further away from the center of the axis, four to five streaks of the same test organism have been found to have an amazing amount of uniformity on the same gradient plate (e.g. only 0.5 mcgs difference on a 10 mcg gradient).

Although not used as a basis for comparative studies, combinations in any desired antibiotic strengths can easily be set up in the gradient plate. In this manner synergisms and antagonisms between antibiotic combinations can be demonstrated. This is an important consideration in therapeutic treatment because of the multiple antibiotics given by the clinicians. Investigator II has included various combinations of antibiotics in the gradient plate along with single antibiotics as a routine procedure in testing for the sensitivity of each potential pathogen.

**Conclusion.** The gradient plate offers to the clinical laboratory a new type of quantitative sensitivity test which is reproducible, inexpensive, and speedily performed. This quantitative test has its limitations in a small laboratory, because of space and equipment, but it can easily be adapted to a large laboratory in which more than four sensitivity tests are performed per day. The gradient is unique in that it can be performed as a routine test, but at the same time, because of its quantitative nature, research studies also can be performed in the study of synergisms and antagonisms. These results may be a direct guide for the clinician in multiple antibiotic treatment.

#### SUMMARY

A review of the basic types of sensitivity tests has been given with a debate between the Titered Disk and Gradient Plate methods. Comparative experimental studies were made within and between two laboratories. These tests were based on the agar plate diffusion method using four sets of commercial disks and the gradient plate method. In the discussion of each method, special attention was placed on the reproducibility and practicality for adaptation into a routine clinical laboratory. The points for and against qualitative and quantitative tests were discussed with an emphasis placed on the need for knowing obtainable *in vivo* antibiotic serum and urine levels.

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#### ANNOUNCEMENT

The Canadian Society of Laboratory Technologists is holding its 1957 convention May 26-30, inclusive, at the Astor Hotel, Vancouver, British Columbia. A most sincere invitation is extended to all members of the American Society of Medical Technologists, and any others interested in the field of medical technology.

Together with a full and entertaining social program, is an extensive and varied scientific program designed to create a wider interest and an increased knowledge and understanding of laboratory work.

## VITAMIN A STUDIES IN CHLORNAPHTHALENE POISONING AND A FEW COMMON DEFICIENCY DISEASES

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In January, 1953, our laboratory was confronted with a problem unique in our experience; it concerned an unknown disease in animals.

At this time the disease seemed more prevalent in cattle. It was characterized in the animals by general weakness, draggy walk, posteria parieses, lacrimation and night blindness. There were pathological changes: keratosis and necrosis of mucous membranes of the gastrointestinal tract and tongue. In advanced stages there was a rapid loss of weight. Abortions were common in pregnancy and death often occurred in ten days to three months.

Since the etiologic agent was unknown it was feared the disease might be transmitted to man either through consumption of the meat and dairy products or through direct contact with the animal itself.

Bacteriologic examinations were made on all body fluids and organs obtained from the affected animal. Urinalysis and hematologic studies were made. All tests were negative.

It was learned a disease with similar symptoms had been observed in Germany as early as 1941. Cattle housed in barns treated with a wood preservative containing chlorinated naphthalene developed peculiar symptoms of "X-disease." Contact with this preservative resulted in mouth and skin lesions, emaciation and a marked decrease in blood carotene and vitamin A levels.<sup>(1)</sup>

One of the most noticeable characteristics of "X-disease" was a vitamin A deficiency. Vitamin A has been called the "anti-infective" or "anti-keratinic" vitamin since the lack of it contributes to a lowered resistance to bacterial infection. A pronounced lack of this vitamin results in replacement of normal epithelium by keratinized epithelium in various parts of the respiratory tract, alimentary tract, eyes and paraocular glands, and the genito-urinary tract.<sup>(2, 3)</sup>

Preformed vitamin A occurs only in lipids of animal origin; the pro-vitamins (carotenes), which are converted into vitamin A are found in plants. Carotenes by an oxidation process in the animal body, are converted into vitamin A.<sup>(4)</sup>

It is now believed that chlornaphthalene has an inhibitory

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effect on the conversion of carotene to vitamin A.<sup>(5, 6)</sup>

Since the symptoms observed in the cattle, by our laboratory, closely resembled those of the German "X-disease"—we, too, should find the decrease in blood carotene and vitamin A levels. If the causative agents were chlornaphthalene derivatives, these agents should be in contact with the animals we were testing.

Blood determination, on cattle, made in our laboratory showed a large percent to have sub-normal carotene and vitamin A levels.

The method for determining carotene and vitamin A levels was that recommended by Dann and Evelyn.<sup>(7)</sup> The two substances were extracted from the serum using ethyl ether after saponification with alcoholic potassium hydroxide. The carotene was measured photometrically at a light absorption band of 440 mu. Vitamin A was measured at 620 mu light absorption after the addition of antimony trichloride.

Since it has been shown that vitamin A is readily destroyed by light radiations,<sup>(4)</sup> the samples to be tested were carefully wrapped in heavy corrugated paper and brought to the laboratory as quickly as possible. If more than an hour was required to reach the laboratory the bloods were refrigerated for transportation. The tests were conducted in amber glassware to protect against light radiations.

The low carotene and vitamin A levels were strongly suggestive of "X-disease," but the causative agent needed to be determined. Since chlornaphthalene had been known to cause the disease, the commercial feeds prepared for cattle were analyzed for the naphthalene derivatives. These tests were made by the Department of Animal Husbandry, A & M College, College Station, Texas.

It was learned the feed pellets prepared commercially contained the chlornaphthalene. To determine the source of the chlornaphthalene the lubricants used on the machinery in processing these feed pellets were analyzed. The lubricants contained the etiologic agent. As the lubricants dripped into the pellet ingredients they were mixed in sufficient quantity to produce the "X-disease." As little as  $\frac{1}{2}$  gram of this chemical ingested each day for ten days can cause the disease in cattle.<sup>(3)</sup>

To further confirm that this disease was characterized by a decrease in carotene and vitamin A levels, three steers, chosen from a typical range herd, were used as controls. The herd's diet had been augmented with commercial feeds. One steer, diseased and showing pronounced symptoms of "X-disease," was used for therapeutic purposes. Two steers, both apparently healthy, were used to produce the disease by feeding them pellets known to contain chlornaphthalene. The condition of the three steers was to be determined solely on their carotene and vitamin A levels.

Carotene and vitamin A determinations were made on each steer twice each week. The diseased steer showed a rapid decrease in both carotene and vitamin A with death occurring in approximately two weeks. Therapy was of no value.

The other steers also showed a decrease in their vitamin levels despite their healthy appearance. A continual decrease in the carotene and vitamin A levels preceded the appearance of clinical symptoms of the disease.

From January, 1953, to January 1956, approximately 1,000 blood determinations were made. During this period when "X-disease" was most prevalent among cattle herds in the Southwest, we have known of only three people who developed symptoms of "X-disease"—and these were mild.

In all three cases the disease was directly attributable to chlornaphthalene in feed pellets. Three ranchers nibbled on the pellets while feeding their cattle. Their symptoms of "X-disease" disappeared when they eliminated cow feed from their diet.

There has been no indication the disease is transmitted from animal to man.

Our tests for decrease in carotene and vitamin A levels over this three-year period have been used to determine the condition of cattle in Texas, Oklahoma, California, Missouri, Mississippi, and Florida. The tests indicate herds may be suffering from a carotene deficiency in range diet or from "X-disease."

Tests indicating "X-disease" have aided ranchers in recognizing the existence of "X-disease" in their herds prior to the appearance of the physical symptoms which presage imminent death of the animal. This early awareness of "X-disease" in their herds has enabled ranchers to save their animals. The prompt elimination from the herd diet of feeds containing chlornaphthalene—and a supplement of vitamin concentrates lead to recovery from the disease.

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## PROTECTION AGAINST RADIATION INJURY BY HETEROLOGOUS TISSUE TRANSPLANTS\*

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The term "radiation injury" is reserved for those manifestations that result from exposure to penetrating radiations such as X rays, gamma rays, fast neutrons, and the like. The effect that occurs in tissue upon exposure to these agents is always one of injury, and a determined effort is being made by many research laboratories to find means by which radiation injury in human beings can be prevented, or can be reversed if it has occurred, as has been done in experimental animals.

As technologists, our professional interest in protection against radiation-induced injury is related to the fact that the number of laboratories concerned with problems related to radiations has increased markedly and many individuals are potentially exposed to radiations of one type or another. Medical technologists are also the ones who take an active part in the clinical laboratory examinations of irradiated individuals.

I shall attempt to bring you up-to-date on matters pertaining to recovery from radiation injury in animals and shall describe briefly some of the studies that are now in progress in our laboratory that are related to radiation recovery.

In 1949, Jacobson<sup>1</sup> first reported the beneficial effect of spleen-shielding on the survival of mice exposed to lethal doses of radiation and showed that no anemia and only a transient leucopenia occurred in the spleen-shielded animals (Figures 1 and 2). Subsequently, in 1951,<sup>2,3</sup> he described the effectiveness of mouse spleen and/or embryo transplants not only on survival (Table 1) but also on the recovery of the blood-forming tissues in mice previously exposed to X radiation. In the same year, Egon Lorenz and his associates<sup>4</sup> reported that approximately 75 per cent of mice survive lethal doses of radiation if they are

**TABLE 1**  
**Effect of Liver or Spleen Cell Suspensions in Survival of Mice Exposed to 900 r**  
**Total-Body X Radiation.**

Mouse Tissue Used	Total No. Cells x 10 <sup>6</sup>	No. Mice Used	28-Day Survival	
			No.	%
Embryo liver cells.....	11.0	12	10	83
	0.20	30	10	33
2-day spleen cells.....	4.0	20	12	60
	0.50	20	11	55
	X radiation only	30	0	0

\* Read before the First North American Conference of Medical Laboratory Technologists, Quebec, Canada, June 1956.

# EFFECT OF SPLEEN SHIELDING ON MORTALITY OF MICE

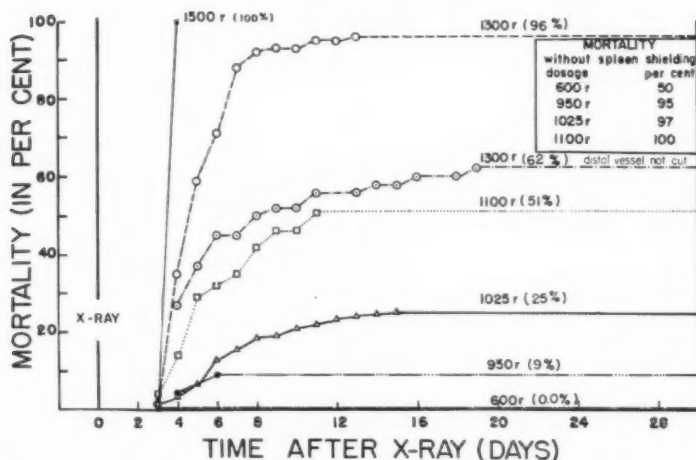


Figure 1. The effect of spleen shielding during X irradiation on the mortality of mice.

injected intravenously after irradiation with bone marrow from normal mice. Jacobson<sup>5</sup> obtained similar results. Attempts have been made to determine whether the nature of the factor(s) responsible for the recovery from radiation injury in animals is due to detoxification or neutralization of toxins produced by radiation; to cellular seeding or regrowth; or to the production of a humoral substance that is supplied by the shielded or injected tissue.

In 1951, Dr. Jacobson<sup>5</sup> demonstrated that regeneration of hematopoietic tissue occurred when spleens of normal baby mice were transplanted intraperitoneally into irradiated rabbits. This was the first work in which heterologous tissue transplants were used successfully. By heterologous, we mean that the donor tissue is obtained from a species of animal that differs from the species of the recipient animal, for example, mouse to rabbit. In the following year (1952), Lorenz and Congdon<sup>6</sup> first reported that the intravenous injection of rat or guinea pig marrow into irradiated mice markedly increased survival. This was corroborated by Jacobson<sup>5</sup> and was also confirmed recently by Cole.<sup>7</sup>

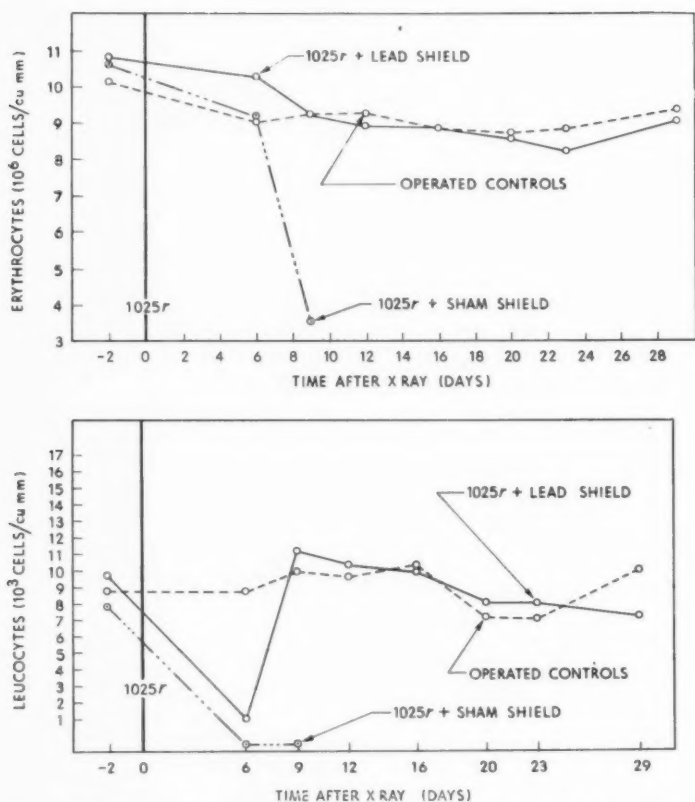


Figure 2. The effect of spleen shielding during X irradiation on the leucocyte and erythrocyte values of mice.

The effectiveness of heterologous transplants (mouse to rabbit, rat to mouse) was thought to be conclusive evidence that the factor(s) responsible for reversing radiation injury is a humoral one. It did not appear likely that heterologous cells would survive in the host beyond the normal survival time of the injected cells nor did it appear likely that the injected cells could seed hematopoietic tissue with cells that repopulate the tissue by multiplication. It seemed more likely that the heterologous tissue lived temporarily and produced a factor(s) that aided recovery. Results of studies reported by Cole *et al.*<sup>8</sup> also appeared to favor the humoral theory. He claimed that only the

nuclei of the cells were intact in the suspensions he used to increase the survival of irradiated mice. Goldwasser<sup>9</sup> was unable to prepare an entirely cell-free suspension using Cole's technique and Jacobson in 1954<sup>10</sup> showed that as few as 50,000 morphologically intact cells were capable of reversing radiation damage. It appeared therefore that beneficial results were obtained only in those instances where living cells were present.

Last year, Jacobson<sup>11</sup> wrote that the intravenous injection of hematopoietic tissue from mice into rabbits previously exposed to 900 r total-body X radiation significantly increased survival of these rabbits. Approximately 60 per cent of the treated rabbits survived as compared with 10 per cent of untreated irradiated rabbits (Table 1). These experiments are described briefly as follows. The tissue used consisted of mouse embryo liver (16- to 20-day), 1-day mouse liver, or baby mouse spleens. Cells obtained from these tissues were suspended in Locke's solution. Cell counts were made according to the usual white blood-cell-counting technique using a hemacytometer and acetic acid. The suspensions were adjusted so that each 10 cc contained the desired number of cells each rabbit was to receive. It was found that  $250 \times 10^6$  cells afforded maximum protection. It is interesting to note that although mouse embryo liver enhances the survival of irradiated rabbits, no such protection was afforded the rabbit when cell suspensions obtained from 21-day rabbit embryo liver were administered, nor was this rabbit embryo tissue effective in increasing the survival of mice exposed to 750 r total-body X radiation.

Recently however Jacobson<sup>12</sup> has found that approximately 68 per cent of irradiated rabbits survive when injected after irradiation with cell suspensions made from the liver of 1-day old rabbits (Table 2) but in a preliminary experiment this tissue had

**TABLE 2**  
**Effect of Mouse or Rabbit Hematopoietic Tissue Cell Suspensions on the Survival of Rabbits Exposed to 900 r Total-Body X Radiation.**

Tissue Used Donor	Total No. Cells $\times 10^6$	No. Rabbits (Recipients)	28-Day Survival	
			No.	%
Mouse liver or spleen.....	200 to 250	37	23	62
Rabbit liver.....	240 to 250	23	15	65
	X ray only	50	5	10
Locke's solution only.....	.....	18	0	0

no significant effect on the survival of mice when 10 to  $20 \times 10^6$  cells were injected intravenously postirradiation. A similar number of cells offers 75 to 100 per cent protection to CF No. 1 mice irradiated with 750 r when the donor cells are obtained from the same strain.<sup>10</sup> Hematologic studies made in a preliminary experiment on irradiated rabbits injected with mouse tissue,<sup>11</sup> in-

dicates that no appreciable effect on recovery of the leucocytes, reticulocytes, or hematocrit values is apparent as compared to irradiated controls. Although this observation suggests that increased survival may not necessarily depend on early regeneration of blood-forming tissue, proof for this statement must await the results of histopathologic studies, especially since a close parallelism exists between survival and hematopoietic recovery in the mouse (Figure 3). Preliminary studies by Jacobson<sup>12</sup> indicate that some beneficial effect is also obtained in rats that received suspensions of mouse hematopoietic tissue after lethal doses of irradiation.

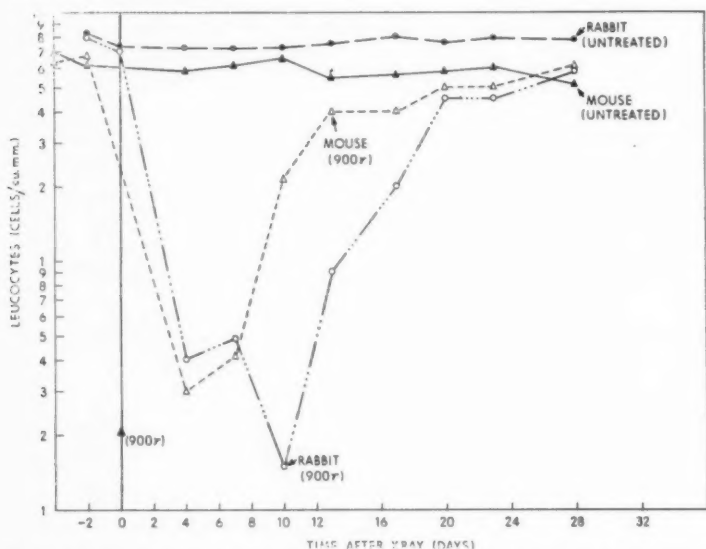


Figure 3. The effect of an injection of a suspension of mouse cells on the leucocyte values of X-irradiated mice and rabbits.

Loutit<sup>13</sup> argues against the benefits derived from suspensions made from tissue of animals of the same species but a different strain (homologous suspensions) because many late deaths occur in irradiated mice injected with such suspensions. It is important to stress that Jacobson<sup>11</sup> found only one death after the 18th day in rabbits injected postirradiation with suspensions made from hematopoietic tissue of mice in experiments that originated 10 months ago.

As stated previously, it was thought that the successful treatment of irradiated animals with heterologous tissue favored the

theory that the factor responsible for the reversal of radiation injury was a humoral one supplied by the injected cells to the irradiated tissue. Until recently the fate of the individual injected cells was not known. That is, it was not known whether cells implant and live a normal life span or whether they function only for a time and then die. Recently Lindsley and associates<sup>14</sup> at Oak Ridge demonstrated, by using marrow carrying an immunogenic marker, that implanted marrow cells contribute as much as 80 per cent of erythrocytes to the peripheral blood of irradiated rats for extended periods of time. This, they state, makes postulation of a humoral theory unnecessary although they admit that the introduced cells may produce (in irradiated animals) some substance that stimulates recovery. Another group<sup>15</sup> working independently at the United States Radiologic Defense Laboratory in California, has found that injected rat marrow cells are able to survive, divide, and repopulate the marrow cavity of irradiated mice, thus permitting the survival of mice exposed to lethal doses of radiation. These workers differentiated rat cells from mouse cells in blood and bone marrow by histochemical procedures and demonstrated that rat cells are strongly positive for the enzyme, alkaline phosphatase, while mouse cells are negative. Loutit and his associates<sup>17</sup> are using chromosomal translocations to determine the fate of injected cells in an effort to shed more light on this controversial subject.

Since cell-free extracts thus far have been proven to be ineffective, it appears that living cells are essential in reversing radiation injury. The mechanism of recovery remains obscure. No treatment is available at present for treating extensive radiation injury in man. Such injury can be controlled adequately only by reducing or eliminating exposure to ionizing radiations. Ritvo and co-workers<sup>16</sup> from Boston have reported in a recent article in the *Journal of the American Medical Association* that serious and even fatal over-exposures to X radiation continue to occur, especially in persons not trained in proper radiologic safety techniques. These severe injuries are frequently the result of carelessness, indifference, or ignorance. Poorly developed safety habits are equally responsible. Excessive exposure to any ionizing radiation is dangerous.

Let us respect this hazard and avoid unnecessary exposure by using the many safety devices that are available today. Furthermore, let us as medical technologists assist in educating those who are unaware of the dangers and above all, let none of us be found amongst those who are careless or indifferent.

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## A REPORT OF C-REACTIVE PROTEIN\*

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### Introduction

In response to a variety of inflammatory stimuli there appears in human blood a protein, not normally present, which may be identified by its capacity to form a precipitate with the somatic C-polysaccharide of the pneumococcus—hence the name C-Reactive Protein (CRP). This protein, believed to be an alpha globulin, was first reported in 1930 by Tillet and Francis,<sup>1</sup> who noted its presence in the acute phase of pneumococcal pneumonia and its disappearance during convalescence. Subsequently, it was isolated and identified by MacLeod and Avery.<sup>2</sup> A specific antiserum has been produced by hyperimmunization of rabbits with repeated injections of purified C-Reactive Protein. Recently, the antiserum has been made available commercially by Schiefel & Company, New York.

The diagnosis of many inflammatory conditions has always presented problems. Although the C-Reactive Protein determination is not specific for any single inflammatory disease or any groups of inflammatory conditions, most investigators seem to agree that it is the most sensitive indicator of an inflammatory process,<sup>3</sup> in many cases more reliable than the erythrocyte sedimentation rate (ESR). Many factors, such as anemia, unrelated to an inflammatory condition, may influence the sedimentation rate making it difficult to interpret. Since the CRP test depends solely upon a single factor, the C-Reactive Protein, which is never found, even in trace amounts, in normal serum, false positives do not occur.<sup>4</sup> Thus there is no "normal range" of values to complicate interpretation of results.

Numerous investigators have reported results of C-Reactive Protein in various diseases. It has been demonstrated in conditions characterized by inflammatory reaction such as rheumatic fever, pneumococcal pneumonia, staphylococcal osteomyelitis, subacute bacterial endocarditis, infections of the colon-typhoid group and streptococcal empyema.<sup>1,3,4,7,9</sup> It has been found in lupus erythematosus,<sup>5</sup> in the majority of cases of rheumatoid arthritis,<sup>8,9</sup> and in myocardial infarction.<sup>9</sup> Havens, Eichman and Knowlton reported that no positive results were obtained in their study of ninety patients in various phases of viral hepatitis.<sup>11</sup> Investigators in evaluating possible serological tests for cancer have reported positive C-Reactive Protein levels in a large majority of patients with well defined cancer. Anderson

\* Scientific Products Foundation Serology First Award, 1956. Read before the First North American Conference of Medical Laboratory Technologists, Quebec, Canada, June, 1956.

and McCarty have reported that changes in amount of C-Reactive Protein tend roughly to parallel changes in the erythrocyte sedimentation rate, but the C-Reactive Protein may be absent from the blood when the sedimentation rate is higher than normal and may be present when the sedimentation rate has returned to normal.<sup>6</sup> Hedlund found that no parallel exists between the white blood count and the presence of the acute phase protein (CRP).<sup>9</sup> All reports agree that C-Reactive Protein determination is the most consistently positive laboratory test in the presence of rheumatic activity.<sup>6</sup>

### Procedure

The test is a simple antigen-antibody reaction in which the antigen formed in the patient's serum (the C-Reactive Protein—if present) combines with the antibody (the C-Reactive Protein Antiserum) to form a precipitate.<sup>4</sup> It is an inexpensive test and is easily and rapidly performed. There is no critical time interval between collection of the blood and performance of the test. The capillary tubes with the precipitate, as well as the original sera, may be kept indefinitely for comparison in serial determinations.<sup>5</sup>

We have followed the technique provided with C-Reactive Protein Antiserum prepared by Schieffelin and Company.

1. Fill approximately one-third of a small capillary tube with the C-Reactive Protein Antiserum.
2. Draw an equal volume of the patient's serum into the tube, being sure that no air bubbles are formed at the point of contact.
3. Clean outside of tube thoroughly with cleansing tissue, invert so that patient's serum is at top of tube, and stand upright in clay.
4. Incubate at 37° C. for two hours. Preliminary qualitative readings may be made at this time. A positive reaction, indicating active inflammatory processes, may be demonstrated within thirty minutes of incubation.
5. Refrigerate overnight.

Final readings are reported as follows:

No visible reaction	0
1 mm. precipitate	1+
2 mm. precipitate	2+
3 mm. precipitate	3+
4 mm. or more precipitate	4+

Note: Both the patient's serum and the C-Reactive Protein Antiserum must be perfectly clear before starting the test. Plasma from oxalated or citrated blood is unsatisfactory. The directions do not mention inactivating the patient's serum. We routinely use serum inactivated at 56° C. for thirty minutes since Anti-Streptolysin O Titers are requested on many of the

same specimens. Parallel C-Reactive Protein determinations on inactivated and non-inactivated serum in our laboratory produced similar readings. The protein will, however, be destroyed by heating above 65° C.<sup>6</sup>

### Results

This report is based upon the first one hundred C-Reactive Protein determinations performed in our laboratory over a period of six months. Results of the tests are correlated with the clinical diagnosis and comparisons of results with the erythrocyte sedimentation rate, the white blood count and Anti-Streptolysin O Titers are given. To date a total of 319 C-Reactive Protein levels have been reported. Of these, 212 have been positive and 107 negative. All of these determinations have been done on hospitalized patients for whom C-Reactive Protein levels have been specifically requested, since the CRP determination is not a routine admission procedure in our hospital. Since the very nature of the group under consideration would suggest a high number of positive CRP levels, we included as a control a group of one hundred individuals for whom we expected negative C-Reactive Protein reactions. Of this group one was positive for CRP, nine revealed a trace, and ninety were negative. The control group was composed of premaritals, pre-employment serologies, food handlers and students. I realize that in such a small group the number of patients which will fall into any particular diagnostic category will be too few to permit a statistical analysis. Table 1 shows the diagnoses represented in our group of one hundred patients and the results of the C-Reactive Protein determinations.

TABLE 1

DIAGNOSIS	Number Patients	Positive CRP*	Negative CRP
Rheumatic Fever and Rheumatic Heart Disease.....	25	21	4
Rheumatoid Arthritis.....	8	7	1
Non-Rheumatic Heart Disease.....	22	20	2
Infections.....	20	18	2
Cancer.....	2	2	0
Osteo-Arthritis, Lupus.....	3	3	0
Miscellaneous.....	20	13	7
Control Group.....	100	10	90

\*CRP-C-Reactive Protein

Note: Those reactions showing a trace of CRP have been included in the positives in the preparation of these tables.

Included in Table 1 were eight patients diagnosed as rheumatic fever. Seven of these were positive for CRP. Six had a diagnosis of myocardial infarction. Four of these had positive C-Reactive Protein reactions. One Lupus erythematosus revealed a trace of CRP. Two patients included in the subgroup listed as

"Infections" were cases of hepatitis—one viral and one chronic. Contrary to the findings of Havens and his associates<sup>11</sup> both were positive for C-Reactive Protein. However, the viral hepatitis was complicated by additional diagnoses of anemia and duodenal ulcer, and the chronic hepatitis by peripheral neuritis.

The figures given in Table 2 are based upon a single erythrocyte sedimentation rate and a single C-Reactive Protein determination performed at approximately the same time. The Wintrobe method was used for the sedimentation rate determinations with normal limits of 0-9 mm. for males and 0-20 mm. for females.

TABLE 2

ESR* and CRP† in 90 Patients	Number Of Patients
Elevated ESR and Positive CRP	68
Elevated ESR and Negative CRP	8
Normal ESR and Positive CRP	7
Normal ESR and Negative CRP	7

\*ESR-Erythrocyte Sedimentation Rate.

†CRP-C-Reactive Protein.

White blood counts were done as a routine procedure on 94 of our group of 100 patients. 10,000 WBC per c.m.m. was used as an arbitrary limit of normal range for the comparison shown in Table 3.

TABLE 3

WBC* and CRP† in 94 Patients	Number Of Patients
WBC less than 10,000 and Positive CRP	50
WBC higher than 10,000 and Positive CRP	30
WBC less than 10,000 and Negative CRP	11
WBC higher than 10,000 and Negative CRP	3

\*WBC-White Blood Count

†CRP-C-Reactive Protein

Anti-Streptolysin O Titers were requested on approximately half of our group of patients with results as shown in Table 4.

TABLE 4

CRP* and AST-O† in 55 Patients	Number Of Patients
Positive CRP and AST-O within normal range	36
Negative CRP and AST-O within normal range	10
Positive CRP and AST-O 224 Units or higher	8
Negative CRP and AST-O 224 Units or higher	1

\*CRP-C-Reactive Protein

†AST-O Anti-Streptolysin O Titer

### Comments

The results of the erythrocyte sedimentation rate and the C-Reactive Protein determination in 90 patients compared closely. The tests appear to be equally sensitive. The C-Reactive

Protein was definitely more sensitive than the white blood count or the Anti-Streptolysin O Titer. There appeared to be no correlation between the C-Reactive Protein and the Anti-Streptolysin O Titer.

### Summary

C-Reactive Protein is described as an "acute phase reactant" which appears in human serum in response to a variety of inflammatory stimuli. A simple, rapid and inexpensive technique for demonstrating the protein is outlined. Our findings in a preliminary group of one hundred patients for whom C-Reactive Protein levels were specifically requested agree with reports previously published indicating that the C-Reactive Protein reaction is non-specific but highly sensitive. It is a sensitive method of detecting inflammatory activity in rheumatic fever patients. It is not a reliable method of differential diagnosis. A comparison of the C-Reactive Protein level with the erythrocyte sedimentation rate, the white blood count and the Anti-Streptolysin O Titers has been made.

This report was prepared in the laboratories of Pittsburgh Branch Laboratory and Presbyterian Hospital.

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## C-REACTIVE PROTEIN: A REVIEW OF ITS DEVELOPMENT AND ITS PRESENT STATUS AS A CLINICAL LABORATORY PROCEDURE

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The first property of C-reactive protein (CRP) became known over a quarter of a century ago. Even though a great deal of work has been done with it since, many technologists and physicians are not familiar with it.

It was noted in 1930 by Tillett and Francis<sup>16</sup> that the serum of patients in the acute stage of lobar pneumonia brought about a precipitate in the presence of the C-polysaccharide of the pneumococci. Although the reagent was derived from the pneumococcus, it was soon learned that its reaction-specificity was not confined to pneumococcal diseases alone.

Before going into the technical and clinical applications of the test, it may be of interest to know something of its development. This can probably best be done by mentioning some of its properties as they became known.

### Properties

First of technical interest is the fact that C-precipitable substance is heat stable up to 65° C.

Of general interest is its distribution among the serum protein components. Early work by Abernathy and Avery<sup>2</sup> consisted of salting out the protein fractions with sodium sulfate, optimally at half saturation. They found C.R.P. present only in the albumin fraction—none present in the globulin fractions. Later with electrophoretic studies, C.R.P. was thought to migrate as an alpha globulin. Stollerman and McCarty<sup>14</sup> found that after defatting and purification, the substance migrates as a beta globulin.

Calcium was found to play a major role in the identification of the substance. Employing a method using potassium oxalate and dialysis, calcium was removed from the serum. Prior to the removal of calcium a four-plus reaction was noted with C polysaccharide. After calcium removal, no reaction occurred. When calcium was restored to the serum in concentration ranging from 0.02 mg. to 0.0003 mg., reactions were obtained from four plus to two plus respectively.

When precipitable substance was washed with dilute solutions of calcium chloride, it remained insoluble. It was then possible to do a quantitative study of precipitable nitrogen—the results of which indicated that the substance was probably protein in nature. As previously stated here this fact was borne out in subsequent investigations.

The properties of the reaction immunologically are seen to be quite different from the usual antigen-antibody reactions. Three

reactions are given below with a brief discussion of each.

1) C polysaccharide + acute phase sera = C.R.P.

This is the first reaction from the work of Tillett and Francis.

2) C.R.P. + Rabbit =  $C_x$ .R.P. + C.R.P.A.

MacLeod and Avery<sup>6</sup> succeeded in producing an antiserum (C.R.P.A.) by injecting rabbits with crystalline C.R.P. Later Wood, McCarty and Slater<sup>18</sup> accomplished the same result employing purified crystalline C.R.P. which they obtained from acites and other serous fluids.  $C_x$ -reactive protein ( $C_x$ .R.P.) found by Anderson and McCarty<sup>9</sup> in rabbit serum is analogous to the human C.R.P. When protein antigens such as human C.R.P. were injected into the rabbit, Wood<sup>17</sup> reports that the production of  $C_x$ .R.P. precluded a high C.R.P. antibody titre.

3) C.R.P.A. + acute phase sera = C.R.P.

This is simply the reaction of the C.R.P. test currently employed in the clinical laboratory. MacLeod and Avery<sup>7</sup> showed that the C.R.P.A. was more specific and more sensitive than was C polysaccharide. C.R.P. was detectable in dilutions of acute phase serum as high as 1:240,000. C polysaccharide determinations were negative with serum dilutions over 1:15,000, C.R.P.A. being therefore, nearly sixteen times more sensitive.

Unlike most antigen-antibody reactions, the antibody is not found in the gamma globulin fraction. Shetlar<sup>12</sup> and others have reported high C.R.P. reactions in cases of agammaglobulinemia.

The appearance of C.R.P. is early in the inflammatory process and disappears shortly after or with the lesion. Stollerman and co-workers<sup>13</sup> found evidence that chemotherapy is the primary cause of the disappearance of C.R.P. with remission of clinical symptoms. In their experiment, salicylates, cortisone or ACTH given several days prior to intravenous injections of typhoid vaccine failed to alter significantly C.R.P. reactions. At the same time the erythrocyte sedimentation rate was suppressed when it was expected to rise.

C-reactive protein is found only in the zoologically related species, man and monkey.

Unlike most antibodies its specificity is not necessarily related to the etiological agent.

Calcium ions must be present.

From the above facts and from recent work published and unpublished there is good reason to believe that the C.R.P. reaction operates independently of antibody production.

### Procedure

Antisera and the few pieces of equipment may be obtained from Schieffelin and Co.

The procedure consists of filling a capillary tube (0.4 mm. x 90 mm.) one-third with antisera and another third with patient's

serum being careful not to cause an air space between the two serums. The tube is placed between the thumb and index finger and allowed to mix by running the two serums up and down the tube four or five times. The tube is placed in a clay based rack with patient's serum uppermost. After a 2-hour incubation period, the rack is refrigerated over night and read. Goldin et al<sup>1</sup> describe a method for obtaining capillary blood for the test. Capillary tubes 2 mm. x 90 mm. are used to obtain blood with a finger puncture as for the blood count. A clay plug is forced into one end and the blood allowed to clot. It is then rimmed with a pipette stylet and centrifuged at moderate speed. The tube is broken a few millimeters from the clot. Serum is then available for use with the smaller capillary tube employed in the usual test procedure.

### Reading

Each tube is wiped with a damp cloth and dried. It has been customary in this laboratory to report both the qualitative and the semiquantitative result, using four plus as the qualitative maximum and the estimated C.R.P. present by direct measurement in millimeters. Thus a four plus reading with 8 mm. will be of more value when a subsequent test, for example, shows a four plus with 4 mm. For more consistent and reliable work it is suggested that the same person, whenever possible, perform the test.

A commercial control sera is available for those who feel they need it.

### Discussion

It is important to remember that the test C.R.P. is a non-specific reaction to inflammation or necrosis. C.R.P. is never found in normal sera and no false positives have been reported. The results of the determinations follow very closely the erythrocyte sedimentation rate (E.S.R.). It is not, however, influenced by any of the following factors which do influence the E.S.R.

#### Factors Influencing E.S.R. Elevations

1. Technical, such as temperature control, inclination and size of tube.
2. Anemia (number, size and shape of RBC).
3. Increased fibrinogen as in pregnancy.
4. Decrease albumen as with nephrotic syndrome.
5. Increased gamma globulin and anemia in convalescent rheumatics.
6. Convalescent stages of other infectious diseases.

It then appears that the C.R.P. is a more certain criterion of an inflammatory process and most certainly an important adjunct to the E.S.R. The C.R.P. becomes positive or normal many times before there is a significant change in the E.S.R.

### Clinical Experience

In bronchial asthma, Aaronson et al<sup>1</sup> found the C.R.P. difficult to evaluate. Upper respiratory infections associated with it play an important role in the production of the abnormal protein.

Roantree et al<sup>10</sup> found consistently positive C.R.P. reactions in bacterial infections, acute rheumatic fever, acute myocardial infarct and metastatic malignancy. Less consistency was found in active tuberculosis, virus infection and active rheumatoid arthritis. It was rarely found in primary carcinoma, superficial dermatitis, uncomplicated chronic leukemia and multiple myeloma.

The following table shows some typical results. The E.S.R. determinations are by the Cutler method. Normal range for men is 0-8 mm. fall per hour, for women 0-10 mm.

SEX	Case	CRP	ESR	ASO	Diagnosis
M C*	1	4 plus or 6 mm	28 mm	Neg.	Salmonellosis
F	2	4 plus or 6 mm	32 mm	—	Urinary inf.
F	3	1 plus or 1 mm	5 mm	—	CA of liver
M	4	neg.	16 mm	—	Cirrhosis of liver
M C	5	4 plus or 4 mm	29 mm	833 u	Rheumatic Fever
F C	6	3 plus or 3 mm	25 mm	633 u	Rheumatic Fever
M	7	1 plus or 1 mm	22 mm	—	Myo-infarct
M	7	1 plus or 1 mm	23 mm	—	Myo-infarct
M	8	4 plus or 4 mm	17 mm	—	Myo-infarct
M	8	2 plus or 2 mm	17 mm	—	Myo-infarct
F	9	4 plus or 4 mm	2 mm	—	Early Hodgkins
F	9	4 plus or 4 mm	26 mm	—	Early Hodgkins

\* C--Child.

When faced with the toxic manifestations of one drug, Stollerman<sup>15</sup> used the C.R.P. test to titrate for the most effective therapeutic agent for treatment of a case of rheumatic fever. The drug which gave the maximum suppression of the inflammatory process was reflected by the C.R.P.

The persistence of C.R.P. during therapy indicates that the dosage is inadequate. On the other hand a negative test does not necessarily indicate the end of the rheumatic process. When therapy is discontinued many patients will again become positive.

In a review of 26 cases of myocardial infarction at a clinical pathology conference at West Suburban Hospital, Dr. J. C. McMillan<sup>6</sup> reported the C.R.P. most significant with the acutely ill patient.

There appears to be much value in the procedure for evaluating and differentiating between cases with borderline EKG's.<sup>11</sup> Simple myocardial ischemia versus acute myocardial infarction show a negative C.R.P. for the former and a strong positive with the latter.

This seems to substantiate earlier work by Hedlund<sup>6</sup> where in 48 cases of myocardial infarction he found 47 positive. Of 11 cases of angina pectoris, 11 were negative. In chronic valvular cardiac diseases, 21 of 21 cases were negative.

There are at the present time more studies in progress with data accumulating for more accurate evaluation of the test both technically and clinically. These findings will give us more information about this test as a tool for the physician.

An example of some of this new work comes from Rapport.<sup>9</sup> He will soon publish a quantitative complement fixation test for C.R.P. which requires little more antisera than that which is used in the current precipitation method. He reports that the amount of C.R.P. reacting at the end point is about 0.02 mg. Studies employing this method have been done on C.R.P. in postoperative patients and also one in cancer which will be finished in a few months.

### Summary

An attempt has been made to briefly review the development of the C.R.P. test. Though most technologists are probably familiar with the procedure, it has been included.

In my opinion the C.R.P. test is a more valuable test as an indicator of an inflammatory and/or necrotic process than is the E.S.R. This is based on the fact that significant results with the C.R.P. test are obtained more consistently and earlier than those of the E.S.R. It is also not necessary to consider normal ranges as any positive C.R.P. result is considered abnormal.

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## ABSTRACTS

### A CLINICAL METHOD FOR THE ASSAY OF SERUM GLUTAMIC-OXALACETIC TRANSAMINASE

Daniel Steinberg, Don Baldwin, and Bernard H. Ostrow (National Institutes of Health, Bethesda, Md.) *J. Lab. Clin. Med.*, 48, p 144-51 (1956).

The present report describes a simple spectrophotometric procedure, modified from that published by Karmen, which can be carried out with use of a relatively inexpensive colorimeter and commercially available reagents. The Bausch and Lomb Spectronic 20 colorimeter was used in most of these studies. One technician can perform 20-30 assays daily and the reagents cost only a few cents a test. The assays are reproducible with a standard deviation of plus-minus 6%.

Standardization in the assay and in reporting of enzyme levels is proposed and some of the properties of the enzyme in normal serum are described. 19 references.

### FLAME PHOTOMETRIC DETERMINATION OF SERUM CALCIUM

Fred L. Hummoller, John R. Walsh, and Marie F. Wharton (Veterans Administration Hospital, Omaha) *J. Lab. & Clin. Med.*, 48, 127-33 (1956).

The proposed method is a modification of that described by Kingsley and Schaffert. In the method described the proteins are precipitated by 6.2% trichloroacetic acid. The filtrate obtained neither causes a turbidity when diluted with the organic solvent nor does it contaminate the burner capillary even after hundreds of samples have been analyzed. The organic solvent used was isopropyl alcohol to a final concentration of 50%. This strength was used because it was found that higher concentrations, while increasing the luminosity, caused flame blowouts; lower concentrations on the other hand reduced the luminosity of the calcium to an undesirable level. The precision and accuracy of the proposed method are adequate and it is readily adaptable to serum samples smaller than 0.5 ml.

### A SIMPLE IMPROVED METHOD FOR THE DETERMINATION OF SERUM IRON II.

Theodore Peters (Veterans Administration Hospital, Boston) *J. Lab. & Clin. Med.*, 48, 280-7 (1956).

This paper describes a simple procedure for serum iron determination. Sensitivity is improved over that of most previous methods by use of the recently introduced iron reagent bathophenanthroline. The tris-iron complex of bathophenanthroline has a molar extinction coefficient more than double that of iron-orthophenanthroline permitting determination of the iron in a 2.0 ml specimen of serum. This improved method is based on the Barkan and Walker procedure.

Two milliliters of serum or plasma (fresh, stored, citrated, oxalated, or heparinized) are treated with 3.0 ml of 0.2N HCl and 1 drop of thioglycolic acid (80% solution in water) at room temperature, the proteins are precipitated by 30% trichloroacetic acid. Four milliliters of aliquot of the supernatant is used and iron is determined by addition of saturated sodium acetate and an alcoholic solution of bathophenanthroline, which is a reagent twice as sensitive for iron as compounds in general use.

This method has been shown to give consistently complete extraction of iron from fresh or frozen serum specimens. Precision is about plus or minus 1 mcg. per 100 ml. It does not involve heating, extraction with organic solvents, daily preparation of reagents or multiple centrifugations. This method is very accurate on cloudy and jaundiced specimens.

#### A METHOD FOR ELUTING ANTIBODIES FROM RED CELL STROMATA

Tibor J. Greenwalt (Milwaukee Blood Center) J. Lab. Clin. Med., 48, p 634-6 (1956).

Existing methods for eluting antibodies from erythrocytes fall into two categories. Those utilizing washed, intact erythrocytes are based on the original procedure described by Landsteiner and employ heat to elute the antibodies. The method which is reported utilizes small volumes of blood and produces hemoglobin-free eluates.

The method described calls for 1.0 ml packed cells washed 5 times with cold saline then adding 0.25% formalin and allow to stand at 4° C for 1 hour. Centrifuge and replace the supernatant with cold distilled water (5 volumes of water and 1/2 volume of toluene). This mixture is agitated on a horizontal shaker for 1/2 hour in the cold. The stromata are separated by filtering three times through a funnel which has been lined with a thin layer of glass wool previously wetted with saline. The stromata concentrated in the cone of the filter is washed with cold saline until the washings show no visible trace of hemoglobin. The resulting preparation is light pink. The cone of wet glass wool is cut off and pushed into a test tube and a volume of 8.0% albumin equal to that of the original packed cells is pipetted into the tube. This is placed in a water bath and heated at 56° C for 10 minutes. The contents are mixed and then aspirated through the glass wool with a pipette while the tube remains in the water bath, and is transferred to a second warm tube. The eluate is clarified by centrifugation at high speed in a heated centrifuge cup and then decanted. It may be used at once or stored at -20° C.

Results are shown in which the stromata elution experiments are compared with parallel Landsteiner eluates. The eluates are described, equal or surpass the Landsteiner preparations in potency. They have the added advantage of being grossly free of heme pigmentation and therefore are expected to be more suitable for electrophoretic and immunochemical studies.

#### A NEW ENRICHMENT MEDIUM FOR CERTAIN SALMONELLAE

F. Rappaport, N. Korforti, and Bty Navon (Municipal Hadassah Hospital, Tel-Aviv) J. Clin. Path., 9, 261-6 (1956).

A new enrichment medium for the cultivation and isolation of Salmonellae from feces is described. The use of this medium was responsible for the detection of almost twice as many cases of salmonellosis as when the two other standard enrichment media were used.

During one year 3,391 stools were examined using the following enrichment media: tetrathionate, sodium selenite and the new enrichment media. After incubation of 16-18 hours a loopful of each of the enrichment media was plated on SS agar. Lactose-negative colonies on SS were inoculated into Kligler's iron agar and urea endol medium. From this examination 120 Salmonellae were isolated. The new enrichment medium effectively prevents the development of intestinal bacteria accompanying the Salmonellae.

The ingredients in the medium, which inhibit the growth of coliform contaminants and permit unrestricted development of Salmonellae, are 4% magnesium chloride and 0.012% malachite green. The medium was found to be superior for enrichment of Salmonellae, with the exception of *S. typhi*, to selenite enrichment broth and the tetrathionate broth.

An important modification of the inoculation procedure is the use of 1:1000 suspension of feces for inoculation of the enrichment medium. This dilution insures freedom from contamination by coliforms and does not decrease the number of isolations of Salmonellae.

#### STUDIES ON THE SPECIFICITY OF AUTO-ANTIBODIES IN ACQUIRED HEMOLYTIC ANEMIA

Leonard V. Crowley and Bertha A. Bouroncle (Ohio State University, Columbus) Blood, 11, p 700-07 (1956).

Twelve patients with hemolytic anemia associated with warm incomplete antibody were studied. In all patients the direct coombs was strongly positive, and free auto-antibody was detected in the serum by the indirect coombs or trypsin technic.

In the twelve patients with acquired hemolytic anemia who were studied, the auto-antibody had no detectable specificity in nine cases, and the remaining three cases showed a detectable specificity.

A significant proportion of patients with autoimmune hemolytic anemia from auto-antibodies of blood group specificity, rather than nonspecific autoantibodies.

Ashby survival studies suggest that in such cases properly selected blood, lacking the antigens against which autoantibodies have been formed, can be transfused successfully.

## ASMT SILVER ANNIVERSARY CONVENTION

Palmer House, Chicago, Illinois

June 23-28, 1957

THEME: GROWTH THROUGH SERVICE

### CHICAGO

Carl Sandburg

Hog Butcher for the World,  
Tool Maker, Stacker of Wheat,  
Player with the Railroads and the Nation's Freight Handler;  
Stormy, husky, brawling,  
City of the Big Shoulders;

In June 1933 Chicago welcomed 43 registrants for the first annual meeting of the American Society of Medical Technologists. Twenty-five years later, in June, 1957 the Illinois Society of Medical Technologists and Chicago hope to welcome close to 2000 medical technologists and friends for our Silver Anniversary. Your convention committee hopes that each and every member of ASMT will seriously consider taking part in our Twenty-fifth Anniversary. The more the merrier! All railroads, air lanes and highways lead to Chicago. Just start out and join us at the Palmer House June 23-28, 1957.

ISABELLE HAVENS

1528 E. 59th St.

Chicago 37, Illinois.

ANNIE LAURIE PEELER

2946 N. Pine Grove Ave.

Chicago 14, Illinois.

*Co-Chairmen of Annual Convention, 1957*

### SCIENTIFIC EXHIBITS

All state societies are invited to present exhibits of their work, interests, activities. Prizes are to be awarded again this year.

Individual exhibits of your work, new techniques, helpful laboratory ideas will also be eligible for prizes.

If you plan to be with us, please send your name and address and the title of your exhibit to Scientific Exhibits Chairman, ASMT Convention, 25 Hermann Professional Building, Houston 25, Texas by April 1, 1957.

### RESERVATIONS

Send all advance reservation and Workshop & Study Group forms to  
ASMT CONVENTION  
25 Hermann Professional Building  
Houston 25, Texas

**Make Hotel reservations direct with the Palmer House.** All blanks will be repeated in the February NEWS RELEASE.

## HOTEL RESERVATION BLANK

Twenty-fifth Annual Convention of the  
American Society of Medical Technologists

June 23-28, 1957

**PALMER HOUSE**  
State and Monroe Streets  
Chicago, Illinois

Please reserve for arrival on \_\_\_\_\_ day \_\_\_\_\_ date \_\_\_\_\_ time \_\_\_\_\_

Check type of accommodation desired

Single	Double Bed (2 persons)	Twin Beds (2 persons)	Suites—2 Rooms (1 person)	Suites—3 Rooms
<input type="checkbox"/> \$ 7.50	<input type="checkbox"/> \$13.50	<input type="checkbox"/> \$15.50	<input type="checkbox"/> \$30.50 and up	<input type="checkbox"/> \$55.00 and up
<input type="checkbox"/> \$ 8.50	<input type="checkbox"/> \$14.00	<input type="checkbox"/> \$16.00	Additional person \$4.00	
<input type="checkbox"/> \$ 8.75	<input type="checkbox"/> \$15.50	<input type="checkbox"/> \$16.50		
<input type="checkbox"/> \$ 9.00	<input type="checkbox"/> \$16.00	<input type="checkbox"/> \$17.00	Dormitory accommodations 4 persons to a room \$4.00 each	
<input type="checkbox"/> \$10.00	<input type="checkbox"/> \$17.50	<input type="checkbox"/> \$17.50		
<input type="checkbox"/> \$11.50	<input type="checkbox"/> \$19.00	<input type="checkbox"/> \$18.00		
<input type="checkbox"/> \$12.50		<input type="checkbox"/> \$19.00		
<input type="checkbox"/> \$14.00		<input type="checkbox"/> \$21.00		

If not able to reserve a room at the rate requested, substitute a room at the next available rate.

Confirm Reservation to: NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_

CITY \_\_\_\_\_

STATE \_\_\_\_\_

Persons sharing the room are listed below \_\_\_\_\_

## SISTERS' HOUSING

Arrangements have been made with the Palmer House to house the Sisters together. This was done in order that as many as possible will be able to stay at the hotel. Please send your reservations to **Sister Hilda Krseminski, St. Francis Hospital, Evanston, Illinois**. Sr. Hilda will then make arrangements for room with the Palmer House. She will also take care of accommodations when convent housing is necessary. Please make your reservations as soon as possible. Be sure to include your date and time of arrival.

## ADVANCE REGISTRATION

This is a **must** in the day and age of so many budget-minded people. It will seem so much less expensive if you try to send a little money before hand by registering in advance. What a relief not to have to stand in line after a long journey to the convention.

Save disappointments by signing up for the workshops, study groups and round table luncheons.

First come first served. Do it now—do not ponder. You will not regret your decision. Use the Registration forms on following pages.

**SILVER ANNIVERSARY CONVENTION**  
**American Society of Medical Technologists**  
**June 23-28, 1957**  
**Palmer House, Chicago, Illinois**  
**ADVANCE REGISTRATION**

Advance registration application and fees will be accepted if mailed on or before May 20, 1957.

Name \_\_\_\_\_

Address \_\_\_\_\_

Local Newspaper \_\_\_\_\_

Number	Event	Price	Total
_____	Registration . . . Member ASMT . . . . .	\$ 5.00 . . . . .	\$ _____
_____	Technical Non-member . . . . .	8.00 . . . . .	_____
_____	Student Non-member . . . . .	5.00 . . . . .	_____
_____	Student Member . . . . .	none	_____
_____	Guest of Member . . . . .	none	_____
_____	Sunday Reception . . . . .	none	_____
_____	Tuesday . . . Edgewater Beach Hotel . . . . .	8.50 . . . . .	_____
_____	Wednesday . . . Round Table Luncheons . . . . .	3.50 . . . . .	_____
_____	Thursday . . . Banquet . . . . .	10.00 . . . . .	_____
_____	Workshops . . . . .	6.00 . . . . .	_____
_____	Study Groups . . . . .	3.00 . . . . .	_____
	Total . . . . .	\$ _____	_____

**IF MORE THAN ONE REGISTRATION, LIST NAMES BELOW**

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Where a registration fee is required, this MUST accompany the advance registration for other events.

**MAKE CHECKS OR MONEY ORDERS PAYABLE TO "ASMT 1957 CONVENTION." MAIL APPLICATION AND FEES TO:**

ASMT CONVENTION  
 25 HERMANN PROFESSIONAL BUILDING  
 HOUSTON 25, TEXAS

**ALL RESERVATIONS FOR ROUND TABLE DISCUSSIONS, WORKSHOPS, STUDY GROUPS, AND OTHER EVENTS MUST BE IN CONJUNCTION WITH ADVANCE REGISTRATION.**

## ROUND TABLE LUNCHEON

Round table discussion on:

Hematology.....	Serology.....
Histology.....	Chemistry.....
Parasitology.....	Blood Bank.....
Bacteriology.....	Mycology.....
Urinalysis.....	

Please indicate your 1st, 2nd and 3rd choices with numbers.

Would you like to talk longer with the morning's speaker?

Or do you have a problem you would like to discuss with an expert?

A chance to discuss your problems or interests, informally, with one of our speakers and experts, will come on Wednesday noon June 26th, at the Round Table Luncheons. Tables of ten will chat over lunch on pre-arranged subjects. General topics are listed above for you to indicate your preferences. Definite table assignments, to accommodate your choice will be arranged at the Convention Registration Desk.

## WORKSHOPS AND STUDY GROUPS

Limited enrollment, Members and Student Members will have priority.

**Study Groups** To be held in the Palmer House. Fee \$3.00. They will consist of demonstrations and lectures.

1. Chemistry—Half-day sessions will be repeated three times.
2. Cytology and Histology—Half-day sessions will be repeated three times. Thursday afternoon for non-delegates only.

**Workshops** To be held under the Palmer House. Transportation and material costs will be included in the work shop fee of \$6.00. Practice will be provided in technical methods.

1. Mycology—One half-day session.
2. Medical Photography—Half-day sessions will be repeated twice on black and white gross photography. Friday morning limited to non-delegates. One half-day session on photomicrography.
3. Bone Marrow and Hematology—One all day session.
4. Tissue Culture—Morning sessions repeated twice.

Check Study Group or Work Shop reservation desired. No reservation will be accepted unless accompanied by Advance Registration Form and payment in full. If a member is elected a delegate, his schedule may be changed accordingly.

<b>STUDY GROUP 1, Chemistry</b> .....	Monday p.m. ....
	Tuesday a.m. ....
	Tuesday p.m. ....
Cytology and Histology.....	Wednesday a.m. ....
	Wednesday p.m. ....
	Thursday p.m. ....
<b>WORK SHOP:</b> 1. Mycology.....	Wednesday p.m. ....

2. Medical Photography
  - Gross Black and White.... Wednesday p.m. ....
  - (non-delegates only).... Friday a.m. ....
  - Microphotography..... Thursday a.m. ....
3. Bone Marrow and Hematology..... Saturday  
June 29
4. Tissue Culture..... Thursday a.m. ....  
8:45-10:15 .....  
10:30-12:00 .....

See the December News Release for more information concerning the Study Groups and Workshops.

